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Maximilian Martin Müllender

Molecular causes for symptom expression of beet necrotic yellow vein virus in *Beta vulgaris*

Molecular causes for symptom expression of beet necrotic yellow vein virus in *Beta vulgaris*

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Maximilian Martin Müllender

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1. Referent: Prof. Dr. Mark Varrelmann

2. Korreferent: Prof. Dr. Stefan Scholten

3. Korreferent: Prof. Dr. Armin Djamei

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List of Abbreviations

A. thaliana	*Arabidopsis thaliana*
A_{405}	Absorption at 405 nm
A. tumefaciens	*Agrobacterium tumefaciens*
A	Adenine
A	Alanine
AA	Amino acid
Acc. No.	Accession number
ACR4	ARABIDOPSIS CRINCKLY 4
AD	Activation domain
AFB1-5	AUXIN SIGNALING F-BOX 1-5
agroinfiltration	*Agrobacterium tumefaciens* mediated leaf tissue infiltration
agroinoculation	*Agrobacterium tumefaciens* mediated inoculation
ALF4	Aberrant Lateral root Formation 4
ANOVA	Analysis of variance
AP	Alkaline phosphatase
ARF	Auxin response factor
AtIAA	*A. thaliana* IAA
AUX1	Auxin transporter protein 1
Aux/IAA	Auxin/indoleacetic acid
AuxRE	Auxin responsive element
Avr	Avirulence determinant
B. macrocarpa	*Beta vulgaris* subsp. *macrocarpa*
B. maritima	*Beta vulgaris* subsp. *maritima*
B. vulgaris	*Beta vulgaris* ssp. *vulgaris*
BaYMV	Barley yellow mosaic virus
BdMV	Burdock mottle virus
BD	Binding domain
BDL	Bodenlos
BiFC	Bimolecular fluorescence complementation
BNYVV	Beet necrotic yellow vein virus
bp	Base pairs
BSBMV	Beet soil-borne mosaic virus
BvIAA	*B. vulgaris* IAA

C. quinoa	*Chenopodium quinoa*
C	Cysteine
C	Cytosine
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CLSM	Confocal laser scanning microscopy
cm	Centimeter
co-IP	Co-immunoprecipitation
CP-RT	CP-read-through
CP	Coat protein
CTD	Carboxyl-terminal dimerization domain
Cys-R	Cysteine-rich
DI	Domain one
DII	Domain two
DIII	Domain three
DIV	Domain four
DAS-ELISA	Double antibody sandwich ELISA
DBD	DNA-binding domain
DOBA	Dropout base agar
dpi	Days post inoculation
dsDNA	Double-stranded DNA
dsRed	*Discosoma* coral RFP
dsRNA	Double-stranded RNA
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EAR	Ethylene response factor-associated amphiphilic repression
ELISA	Enzyme-linked immunosorbent assay
ET	Ethylene
EXP	Expansin
F	Phenylalanine
fw	Forward
G	Glycine
G	Guanine
Gal	Galactose

Glu	Glucose
GFP	Green fluorescent protein
GOI	Gene of interest
H	Histidine
HA	Influenza hemagglutinin
HDAC	Histone deacetylase
HR	Hypersensitive response
I	Isoleucine
IAA	Indoleacetic acid
ICK/KRP2	Inhibitor-Interactor of CDK/Kip Related Protein2
IDR	Intrinsically disordered region
IgG	Immunoglobulin G
JA	Jasmonic acid
K	Lysine
kDa	Kilodaltons
KEGG	Kyoto Encyclopedia of Genes and Genomes
L	Leucine
LAX	Auxin transporter-like protein
LBD/ASL	Lateral Organ Boundaries-Domain/Asymmetric Leaves2-like
LR	Lateral root
M	Methionine
MILV	Magnifera indica latent virus
MP	MONOPTEROS
miRNA	Micro RNA
mRFP	Monomeric RFP
mRNA	Messenger RNA
N	Asparagine
NBT/BCIP	Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3'-indolyphosphate
NES	Nuclear export signal
NLS	Nuclear localization signal
O. sativa	*Oryza sativa*
O-GlcNAcylation	*O*-linked β-*N*-acetylglucosamination
OD	Optical density
OD_{600}	Optical density at 600 nm

ORF	Open reading frame
OsIAA	*O. sativa* IAA
P. betae	*Polymyxa betae*
P	Proline
PB1	Phox/Bem1p domain
PCR	Polymerase chain reaction
PIN	Pin-formed protein
PPV	Plum pox virus
RT-qPCR	Reverse transcriptase quantitative PCR
R	Arginine
Raf	Raffinose
RBSDV	Rice black streaked dwarf virus
RdRp	RNA-dependent RNA polymerase
RDV	Rice dwarf virus
REP	Replicase protein
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RSMV	Rice stripe mosaic virus
RSNV	Rice stripe necrosis virus
RSV	Rice stripe virus
RT-PCR	Reverse-transcription PCR
rv	Reverse
SA	Salicylic acid
SBR	Syndrome Basses Richesses
SBWMV	Soil-borne wheat mosaic virus
SCFTIR1/AFBs	SKP1-CULLIN1-F-BOX (SCF)-ubiquitin ligase complex
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sec	Second(s)
SLR	Solitary-root
smRSGFP	Soluble-modified red-shifted GFP
SRBSDV	Southern rice black streaked dwarf virus
ssDNA	Single-stranded DNA
ssRNA	Single-stranded RNA

SV40	Simian-virus 40
T	Threonine
T	Thymine
TBV	Tulip breaking virus
TGB	Triple gene block
Ti	Tumor-inducing
TIR1	TRANSPORT INHIBITOR RESPONSE 1
TMV	Tobacco mosaic virus
ToCV	Tomato chlorosis virus
TPL/TPR	TOPLESS and TOPLESS-RELATED co-repressors
TRV	Tobacco rattle virus
U	Uracil
V	Valine
VIGS	Virus-induced gene silencing
W	Tryptophan
w/v	Weight/volume
WhSMV	Wheat stripe mosaic virus
wt	Wild type
x	Any amino acid
XPP	Xylem pole pericycle
Y2H	Yeast two-hybrid
Y	Tyrosine

1. Introduction

Besides plant diseases caused by fungi, bacteria and animal pests, there are a lot of viral diseases. The economically and scientifically most important plant viruses are tobacco mosaic virus, tomato spotted wilt virus, tomato yellow leaf curl virus, cucumber mosaic virus, potato virus Y, cauliflower mosaic virus, African cassava mosaic virus, plum pox virus, brome mosaic virus and potato virus X (reviewed in Scholthof *et al.*, 2011). Almost all crops can be infected and damaged by viruses including sugar beet. Important viral species, in sugar beet are beet curly top virus (BCTV), beet mild yellowing virus (BMYV), beet yellows virus (BYV), beet mild yellowing virus (BMYV), beet chlorosis virus (BChV), beet mosaic virus (BtMV) and beet necrotic yellow vein virus (BNYVV). These diseases can affect plant development, including sugar accumulation, and thus can cause significant economic damage to sugar production.

Like human or animal viruses, plant DNA viruses are intracellular parasites that rely on the host's replication machinery to reproduce. RNA viruses encode their own polymerase, an RNA-dependent RNA polymerase (RdRp). Next to a few virus families with double-stranded RNA (dsRNA), single-stranded DNA (ssDNA), or double-stranded DNA (dsDNA) genomes, most known plant viruses contain a small single-stranded RNA (ssRNA) genome in positive orientation. Most plant viruses are multipartite and encode 4–10 gene products responsible for replication, transmission by vectors, distribution within the host plant, and specific host-virus interactions (reviewed in Hull, 2002). Since most plant viruses have an RNA genome, an RdRp is essential for successful replication. Therefore this unique class of nucleic acid polymerases is encoded in the genome of RNA viruses (reviewed in Jia & Gong, 2019). Furthermore, RdRps are responsible for the high mutation rate of viruses and consequently for rapid evolutionary adaptation as a consequence of the lack of proofreading activity (reviewed in Elena *et al.*, 2008). The mutation rate from RNA viruses ranges approximately between 10^{-6} to 10^{-4} substitutions per nucleotide site per cell infection (s/n/c), whereas DNA viruses have a much lower rate of 10^{-8} to 10^{-6} s/n/c (reviewed in Duffy, 2018; Peck & Lauring, 2018). The high mutation rate and short reproduction time cause an enormous complexity and flexibility of viruses, which leads to a very fast evolutionary adaptation process to ensure an infection of the host that is beneficial for the virus.

Besides the horizontal transmission of viruses, for example when the embryos in the seeds are already infected with the virus by the infected mother plant, there exists vertical transmission of the virus from an infected to a healthy host (reviewed in Singh *et al.*, 2020). Unlike animal viruses, plant viruses cannot invade host cells by endocytosis. Before a virus can infect a plant and release its genomic components, the cell wall must first be overcome by mechanical injury to the plant tissue. Plant viruses are therefore transmitted only by external agents such as insect vectors, fungal vectors, parasitic plants (*e.g. Cuscuta*) or human activities via contaminated equipment (reviewed in Jeger, 1998; Hull, 2002; Singh *et al.*, 2020). The virus–vector relation with insects can be divided into three transmission modes: nonpersistent, semipersistent and persistent (reviewed in Power, 2000). Viruses that are only carried on the insects' mouthparts are called 'nonpersistent', they are only present on the stylet and are not incorporated into the vector. 'Semipersistent' viruses are taken up into the foregut of the vector and 'persistent' viruses even pass through the vector and invade into the hemolymph of the insects. Non-persistent viruses can only be transmitted for a short time, whereas semi-persistent and persistent viruses remain infectious in the vector for a long time and can infect hosts over the salivary glands (reviewed in Power, 2000). For fungal vectors, transmission is distinguished based on virus acquisition and the location of virions relative to the resting spore. *In vitro* acquisition means that virions are adsorbed on the surface of the zoospores and not taken up into the resting spores. During *in vivo* acquisition, virions are incorporated into the thallus of the fungal vector and the virus is located within resting spores, in which the virus remains infectious for a long time (reviewed in Campbell, 1996).

The coat or capsid protein (CP) is mainly responsible for virus transmission and spreading. This essential component of plant viruses encapsidates viral genomic nucleic acids, as the name implies. However, CPs are multifunctional, which means they are responsible for other important functions such as pathogenicity, infectivity, distribution within the plant and mode of transmission (reviewed in Callaway *et al.*, 2001). CPs play an important role in the transmission of viruses by vectors. These proteins can either interact directly with receptors inside the vector or indirectly via helper proteins (Ng & Falk, 2006; Ng & Zhou, 2015; Whitfield *et al.*, 2015; Agranovsky, 2021). In addition, CPs are the major determinants for the viral shape. Most plant viruses have an elongated helical structure that is either rod-shaped, such as tobacco mosaic virus (TMV), or filamentous like potato virus Y (PVY) (reviewed in Lacomme &

Jacquot, 2017; Bak & Emerson, 2020; Evtushenko *et al.*, 2020). Additionally, some virions are icosahedral and divided into bacilliform virions such as rice tungro bacilliform virus (Cheng *et al.*, 1992). Twin virions compose of two joined incomplete icosahedra and are common among members of the family *Geminiviridae* (reviewed in Evtushenko *et al.*, 2020; Shafiq *et al.*, 2020).

Summing up, plant viruses are extremely diverse in their shape and transmission. These organisms have already been detected in all agriculturally important plant species, but also in a lot of ornamental plants. However, they do not always cause economic damage. The tulip breaking virus (TBV), for example, causes a very beautiful, non-lethal colour-breaking of tulip flowers that was highly sought in the 17th century, leading to peak prices for tulip bulbs ("tulipomania") (reviewed in Garber, 1989). Just as diverse as the host range and symptoms caused by plant viruses is the genetic diversity of viruses. As mentioned before, genes of viral proteins have a high mutation rate due to the error-proneness of viral RdRps. And yet viruses are very simple with 4–10 gene products. Despite this simplicity, viral proteins are often multifunctional to fulfil the requirements for replication, movement and symptom development, meaning that viral proteins often have an extensive network of cellular interaction partners that has been developed during the co-evolution of viruses and their hosts (reviewed in Callaway *et al.*, 2001; Nagy, 2016; Valli *et al.*, 2018).

1.1 Beet necrotic yellow vein virus

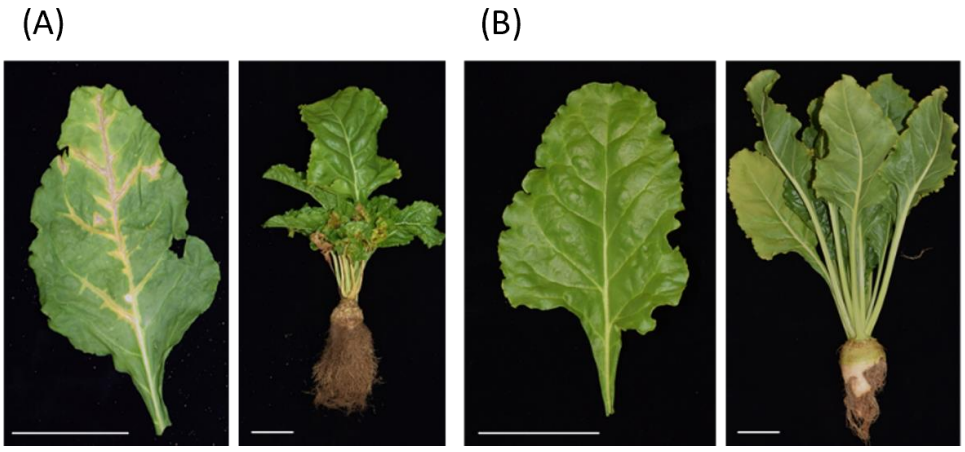

Figure 1) Phenotypes of **(A)** a beet necrotic yellow vein virus infected sugar beet vs. **(B)** a non-inoculated, healthy (mock) sugar beet. The plants were mechanically inoculated into the hypocotyl, and the pictures were taken 69 dpi (Scale bar = 5 cm).

Rhizomania is considered as the most important disease of sugar beet worldwide. The causal agent of this disease is beet necrotic yellow vein virus (BNYVV). It was first described in Italy in the early 1950s and spread to almost all sugar beet-growing areas in the following decades (reviewed in McGrann *et al.*, 2009). Under greenhouse conditions, the disease causes symptoms on leaves, such as yellowing and necrosis along the veins that are rarely observed under field conditions (reviewed in Peltier *et al.*, 2008). More important, however, are the severe symptoms of the infected roots, such as reduced size, wineglass shape, necrosis of the vascular tissue and the massive proliferation of the lateral roots (LRs), manifested as root beard symptom (reviewed in Peltier *et al.*, 2008) (Figure 1). These root symptoms leading to dramatic reduction of taproot weight and massive sugar yield losses of up to 80%, making BNYVV economically the most important viral pathogen in sugar beet cultivation (reviewed in Peltier *et al.*, 2008; McGrann *et al.*, 2009). Such economic losses underline the importance and necessity of studying this disease in detail. BNYVV belongs to the genus *Benyvirus* within the family *Benyviridae*. Next to BNYVV three other viruses belong to this genus, namely beet soil-borne mosaic virus (BSBMV), rice stripe necrosis virus (RSNV) and burdock mottle virus (BdMV) and two putative members: magnifera indica latent virus (MILV) and wheat stripe mosaic virus (WhSMV) (Gilmer *et al.*, 2017). BNYVV and BSBMV possess a similar genome organization and both viruses can infect *Beta vulgaris*. In addition to a high sequence similarity of the genomic components (Section on BNYVV pathotypes under 1.1.3), it can be assumed that these viruses are the closest relatives within the benyviruses (Laufer *et al.*, 2018b). In contrast to BNYVV, BSBMV does only occur in the US and does not cause any significant economic damage to sugar beet cultivation, as infected roots mainly remain asymptomatic (Wisler *et al.*, 2003).

1.1.1 Vector transmission of BNYVV

BYNVV as well as BSBMV are naturally transmitted by the soil-borne plasmodiophoromycete *Polymyxa betae* Keskin through infection of LRs (Keskin, 1964; Tamada & Kondo, 2013). This vector is an obligate intracellular parasite of sugar beet roots and belongs to the family *Plasmodiophoromyceae* within the monophylum Cercozoa (Irwin *et al.*, 2019). Natural hosts for *P. betae* are almost all members of the *Amaranthaceae*, including the subfamily *Chenopodiaceae* (Keskin, 1964; reviewed in Simpson, 2018) but also some members of the

Portulacaceae (Abe & Ui, 1986; Mouhanna *et al.*, 2008). Most members of the *Amaranthaceae* can also be naturally infected by BNYVV, such as *Beta macrocarpa* and *B. vulgaris* (Tamada *et al.*, 1989; Tamada & Abe, 1989; Hugo *et al.*, 1996; Yanar *et al.*, 2005). However, some experimental hosts can also be artificially infected such as *Chenopodium quinoa*, *Spinacea oleracea, Tetragonia expansa* or *Nicotiana benthamiana* (reviewed in McGrann *et al.*, 2009). For transmission of BSBMYV and BNYVV through *P. betae*, LRs of young *B. vulgaris* plants are infected by zoospores originating from zoosporangia or sporosori (Ciafardini, 1991). Zoospores infect the host cells by encystement at the host cell wall, development of a tubular structure and penetration of the cell through an adhesive outgrowth (adhesorium/appresorium) with a dense dagger-like body (reviewed in Kanyuka *et al.*, 2003). After nuclear multiplication within the plant cell (plasmodium), the nuclei are enclosed in secondary zoospores. At this stage virus particles are released into the plant cell and new viruses are uptaken into zoospores (*in vivo* acquisition). These zoospores are then released by exit tubes either outside of the root, or into the adjacent root cells (reviewed in Littlefield *et al.*, 1998; Kanyuka *et al.*, 2003). Secondary zoospores can either initiate the generation of a new plasmodium, resulting in more secondary zoospores or develop into sporogenic plasmodia, where resting spores are formed. Once the field is infested by *P. betae*, the resting spores as well as the possibly contained viruses remain viable in the soil for years (Tuitert, 1991, 1993b, 1993a). Therefore BNYVV transmission can be classified as *in vivo* acquisition. Since biological control of *P. betae* is not very successful and effective (Naraghi *et al.*, 2014), rhizomania resistant sugar beet varieties are used to reduce economic damage (Section on Resistance to control BNYVV in field under 1.1.4).

1.1.2 Genome organization

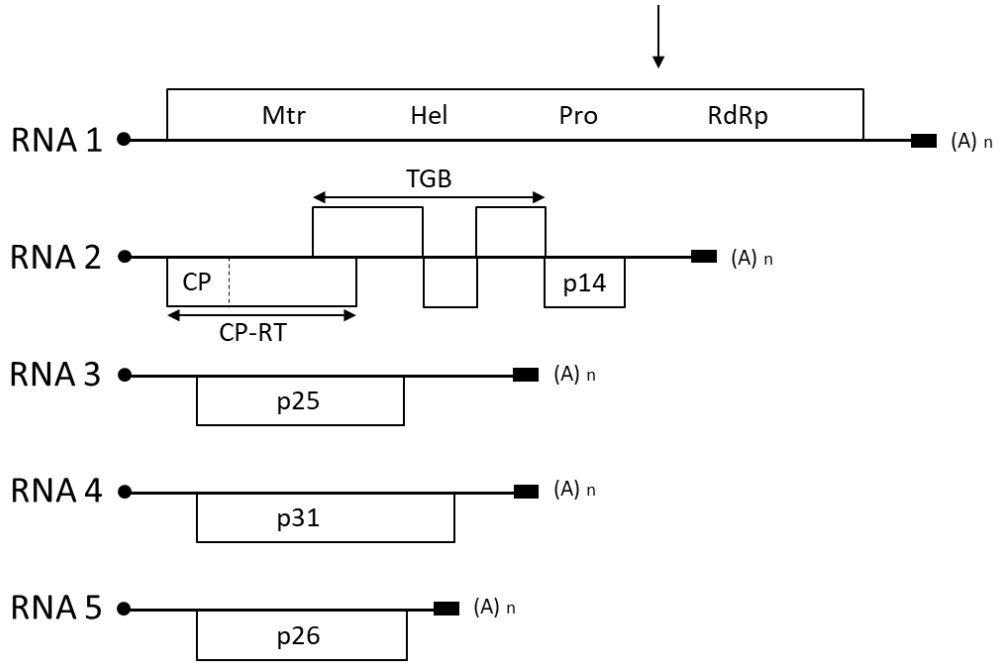

Figure 2) Genomic organization of beet necrotic yellow vein virus, consisting of four or five RNAs (RNA1-5). The 5' cap structure is indicated as black dot and the poly A-tail [(A) n] at the 3' end is shown as square. Open reading frames are shown by boxes with resulting protein. The black arrow above RNA1 indicates autocatalytical cleavage of one protein into two smaller proteins. One protein contains motifs for methyltransferase (Mtr), helicase (Hel) and a papain-like protease (Pro) and the other protein is the RNA-dependent RNA polymerase (RdRp). (CP = coat protein, RT = read-through protein, TGB = triple gene block). (Modified after Ward *et al.*, 2007; Peltier *et al.*, 2008; Gilmer *et al.*, 2017).

The genome of BNYVV consists of four to five positive-sense, single-stranded RNAs. Each RNA is capped at the 5' end and polyadenylated at the 3' end (Figure 2). RNA1 possess one open reading frame (ORF) encoding a large protein that is autocatalytically cleaved into two smaller proteins. One protein contains motifs for methyltransferase, helicase and a papain-like protease and the other protein is the viral RdRp (Bouzoubaa *et al.*, 1987; reviewed in Richards & Tamada, 1992). RNA2 possesses six ORFs, encoding a coat protein (CP), terminated by a suppressible UAG stop codon, a CP-read-through (CP-RT) protein, a triple gene block (TGB) for cell-to-cell movement and a small 14 kDa cysteine-rich protein (p14) acting as silencing suppressor (Tamada & Kusume, 1991; Dunoyer *et al.*, 2002). RNA3 encodes the pathogenicity factor of BNYVV, p25, which is responsible for symptom development (Tamada *et al.*, 1989). Furthermore, p25 has been associated with *Rz1* resistance-breaking (Section on Resistance to control BNYVV in field under 1.1.4). Additionally, RNA3, more exactly the core region, is important for systemic infection and vascular movement in *Beta* species (Lauber *et al.*, 1998; Flobinus *et al.*, 2018).This non coding RNA (ncRNA), which is processed by Xrn1, a 5'-to-3'

exoribonuclease, seems to act synergistically with p14 encoded on RNA2 (Flobinus *et al.*, 2018). RNA4 is mainly involved in the successful transmission by *P. betae* but also enhances the symptom development (Tamada & Abe, 1989; Rahim *et al.*, 2007). Nevertheless, RNA4 is not necessary for mechanical virus infection and propagation in *N. benthamiana* or *Beta* species (Wu *et al.*, 2014). A fifth RNA occurs in BNYVV P-type, encoding a 26 kDa protein (p26) (Koenig *et al.*, 1997) (Figure 2) (Section on RNA5 encoded p26 as pathogenicity factor of BNYVV P-type under 1.3). It can be concluded, that RNA1 and RNA2 are essential for virus replication and infection whereas RNA3, RNA4 and RNA5 are involved in pathogenicity and vector transmission (reviewed in Richards & Tamada, 1992). Therefore, RNA3-RNA5 are assumed to enhance the viral efficacy in terms of infection and propagation.

1.1.3 BNYVV pathotypes

Depending on the composition and sequence of the four or five RNAs, BNYVV is divided into three types, A-, B-, and P-type. These types can be differentiated by sequence differences of the *CP* and *p25* gene of RNA2 and 3, respectively (Schirmer *et al.*, 2005). In addition, the P-type can be distinguished from A- and B-type by the presence of RNA5 (Koenig *et al.*, 1997). Some BNYVV isolates from Asia can also carry an additional RNA, named J-type RNA5 (Tamada *et al.*, 1989). However, quite high nucleotide sequence differences (8.4%) were detected in the coding region between P- and J-type RNA5 (Koenig *et al.*, 1997; Miyanishi *et al.*, 1999). Therefore, both RNA5 types must be clearly distinguished. Unlike the P-type, which differs from the A-type in *CP* sequence, the isolates carrying the J-type RNA5 cannot be separated from the A-type or the B-type (approx. 93% sequence identity) (Miyanishi *et al.*, 1999; Chiba *et al.*, 2011).

Geographically, the A-type spread in nearly every growing region including most European countries as well as in the US, China, Iran and Japan (Saito *et al.*, 1996; Schirmer *et al.*, 2005; Borodynko, 2006; Mehrvar *et al.*, 2009). The B-type is present mainly in Northern Europe and China (Koenig & Lennefors, 2000; Schirmer *et al.*, 2005; Borodynko, 2006). The P-type is the least common type and was only reported in France (Pithiviers) (Koenig *et al.*, 1997), Kazakhstan (Koenig & Lennefors, 2000), the UK (Harju *et al.*, 2002; Ward *et al.*, 2007) and Iran (Mehrvar *et al.*, 2009) so far. Japan and China are the only countries where J-type RNA5 could be detected in the field (Tamada *et al.*, 1989; Kiguchi *et al.*, 1996).

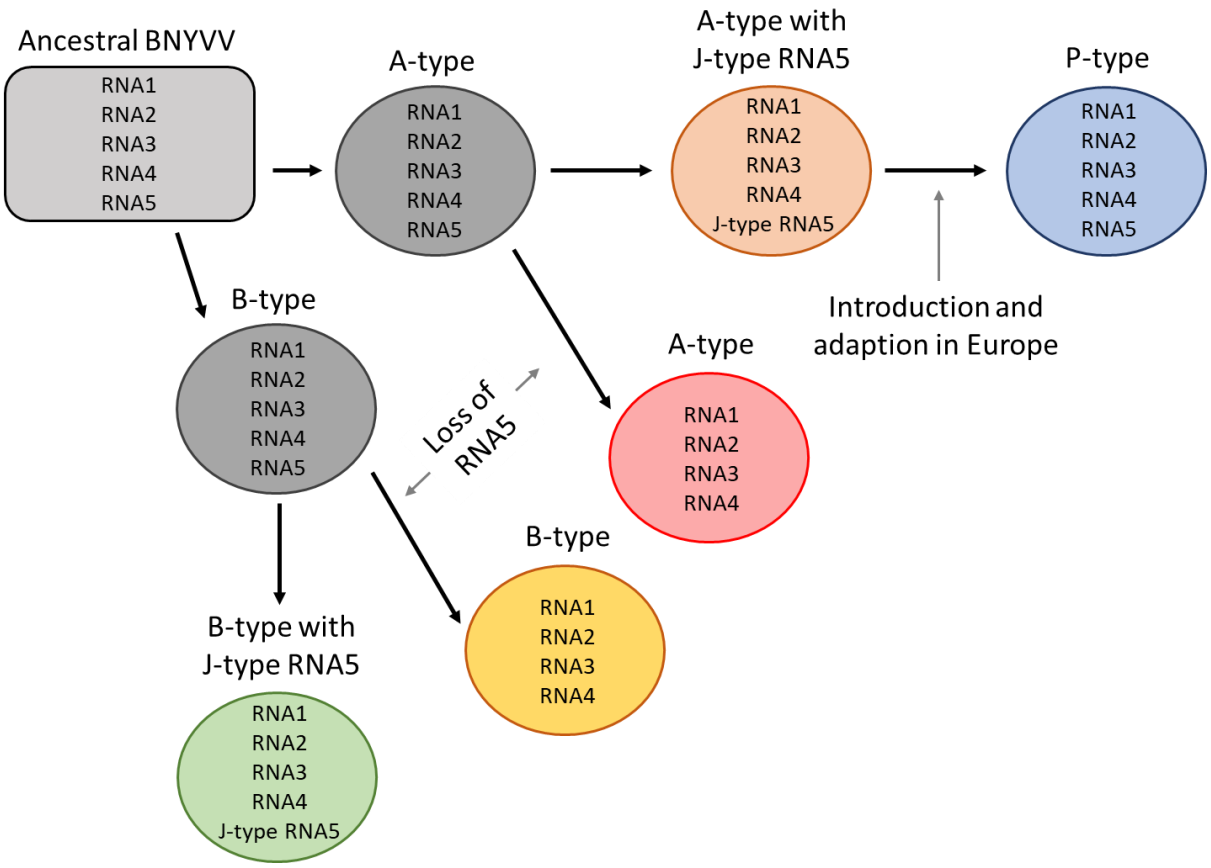

Figure 3) Evolutionary steps of beet necrotic yellow vein virus types derived from one BNYVV ancestor population with five RNAs. The genome composition is listed within the ovals and the respective BNYVV pathotype is given above each oval. This classification into A-, B- and P-type is based on the coat protein gene sequence. Black arrows indicate major lineages and grey arrows indicate evolutionary events. (Modified after Nakagami *et al.*, 2021 and Chiba *et al.*, 2011).

Based on sequence comparison of the *CP* gene of the different BNYVV types and their geographic distribution, a hypothesis on the phylogenetic relationship of the pathotypes was created (Figure 3) (Chiba *et al.*, 2011; Nakagami *et al.*, 2021). Due to strong sequence differences in RNA2 of the A- and B-type, it is hypothesized that these pathotypes evolved early from one ancestral BNYVV population with five RNAs. These pathotypes, then spilt into two subgroups, A- and B-type without RNA5 and A- and B-type with RNA5 which was named J-type RNA5 (Figure 3) (Schirmer *et al.*, 2005; Chiba *et al.*, 2011; Nakagami *et al.*, 2021). Based on the RNA2 sequence, the P-type appears to be more closely related to the A-type (Koenig & Lennefors, 2000; Schirmer *et al.*, 2005). For this reason it was hypothesized, that the P-type was introduced to France as A-type with J-type RNA5 from Asia through soil adhering to mulberry tree plantlets imported for multiplication and used for feeding silkworms and

adapted to the local conditions (Meulemans *et al.*, 2003). As mentioned above, the gene sequences of p26 are quite different between the J- and P-type (Miyanishi *et al.*, 1999).

Apparently, it is difficult to make clear statements about the evolutionary relationship of the pathotypes, since viruses are often subject to vertical gene transfer. This is accomplished either by homologous or non-homologous recombination of DNA/RNA fragments or by replacement of entire genetic components, which is referred to as reassortment. For multipartite viruses such as BNYVV this means that RNAs can be mixed when two virus strains infected the same cell. This leads to new combinations. For BNYVV, this means that RNAs are mixed that were originally assigned to other pathotypes, mainly this is described between the A- and B-type (Ward *et al.*, 2007; Li *et al.*, 2008; Koenig *et al.*, 2009a; Nakagami *et al.*, 2021), but there are also reports of reassortants with the P-type (Yüksel Özmen *et al.*, 2020). However, it must be stated here that most studies are based on field populations, where it is difficult to discriminate between reassortants and mixed infections (Galein *et al.*, 2018). To make clear statements about which reassortants are possible and viable and which biological properties they exhibit, an experimental system must be generated, such as an infectious cDNA clone in which all RNAs can be exchanged with each other.

1.1.4 Resistance to control BNYVV in the field

As mentioned before, there is no efficient measure known to control the vector *P. betae* in field. The most efficient way to control rhizomania disease is the cultivation of resistant sugar beet varieties. The best-known principle in breeding to generate resistant varieties is the use of resistance genes. Such genes are plant genes, that confer resistance to viruses, bacteria, fungi and even nematodes. Among various other virus resistance mechanisms and pathways in plants, one possibility of resistance is the activation of a defence response. Simplified, resistance genes have two essential functions during this mechanism: to recognize the pathogen and to initiate the defence response, such as hypersensitive response (HR) (reviewed in Soosaar *et al.*, 2005). Proteins of the pathogens, so called Avr (avirulence) determinants, are recognized by the resistance protein. This recognition is either direct or with the help of plant proteins (reviewed in van der Biezen & Jones, 1998; Dangl & McDowell, 2006; van der Hoorn & Kamoun, 2008). Molecular analysis of related *Beta* species and extensive breeding work have led to the development of resistant sugar beet varieties and to the

identification of resistance genes against BNYVV. The first efficient gene, used for rhizomania control was the gene *Rz1*, which was introduced in the 1980s (reviewed in Scholten & Lange, 2000). In the following years, more and more genes were added, such as *Rz2*, *Rz3*, *Rz4* and *Rz5* (reviewed in Biancardi & Tamada, 2016). Most genes do not confer complete resistance, they reduce damage and symptom expression. Since low virus replication still occurs when the roots of resistant varieties are naturally infected, the virus inoculum potential is maintained in the soil (Pferdmenges, 2007). Nowadays, *Rz1* and *Rz2* are the only resistance genes which are used economically (reviewed in Scholten & Lange, 2000). These genes mediate a partial resistance reducing virus multiplication and preventing symptom development. This means that economical sugar beet production is still possible even in BNYVV-infested fields.

Breeders make use of related, sexual compatible plant species when generating resistant varieties, as in the case of *Rz2* in *B. vulgaris* ssp. *maritima*. These naturally resistant plant species are crossed into commercial sugar beet varieties to transfer the resistance to sugar beet. In most cases, no genes or mutations are selected to be introduced into the plant. Often, desired traits such as a resistance are selected to be transferred without knowing the genetic background. Simplified, this means that related species with the desired resistance traits are selected for crossing to transfer the resistance to the crop (reviewed in Allard, 1999). In the case of sugar beet, only the sequence of *Rz2* has been uncovered so far (Capistrano-Gossmann *et al.*, 2017), not the sequence of *Rz1*. Although the exact identity and sequence of *Rz1* is not known, it is assumed that the pathogenicity factor p25 might be the Avr determinant of this resistance (Koenig *et al.*, 2009b; Bornemann *et al.*, 2015; Liebe *et al.*, 2020). Just recently, Wetzel and coworkers demonstrated that BNYVV TGB1 represents the Avr determinant of *Rz2* (Wetzel *et al.*, 2021).

1.1.5 BNYVV resistance-breaking

One problem sugar beet growers have been facing since the beginning of the 21th century are *Rz1* resistance-breaking BNYVV isolates (Liu *et al.*, 2005b). These isolates were shown to appear in the Imperial Valley in the USA for the first time where resistant plants showed strong rhizomania symptoms resulting in strong economic losses (Liu *et al.*, 2005b). The resistance-breaking ability of these virus isolates is based on the fact that the resistance

protein no longer recognizes the Avr determinant (reviewed in Luderer & Joosten, 2001). Previous studies indicate, that *Rz1* resistance-breaking is mediated by amino-acid changes a hypervariable region between p25 amino acids 67-70 (tetrad) (Acosta-Leal & Rush, 2007; Acosta-Leal *et al.*, 2008; Pferdmenges *et al.*, 2008; Acosta-Leal *et al.*, 2010). Single amino acid exchanges in this motif mediate *Rz1* resistance-breaking, at least for the A-type (Koenig *et al.*, 2009b; Bornemann *et al.*, 2015; Liebe *et al.*, 2020). Another possible resistance-breaking mechanism that has been proposed is the presence of an additional genetic compound, RNA5. In laboratory tests, BNYVV has been shown to replicate to higher levels in *Rz1* resistant plants when the BNYVV type carries a fifth RNA, as evidenced by the fact that higher ELISA values were detected (Tamada *et al.*, 2020). Tamada and coworkers could not find evidence in their studies that BNYVV isolates from Japan, carrying J-type RNA5 can overcome *Rz2* via natural infection. In contrast, infected *Rz2* resistant plants have been found in the Pithiviers area of France, but the roots did not display the characteristic root symptoms and the resistance-breaking properties of the BNYVV isolates were not confirmed in greenhouse studies (Galein *et al.*, 2018). Since no resistance-breaking of *Rz2* had been reported to date, control of BNYVV in the future will rely on this resistance gene. Regarding the P- and J-type RNA5, both genomic components differ based on their sequence and distribution (Miyanishi *et al.*, 1999). As mentioned above, the J-type RNA5 was exclusively found in Japan and China (Tamada *et al.*, 1989; Kiguchi *et al.*, 1996) whereas the P-type was detected in France (Pithiviers) (Koenig *et al.*, 1997) , Kazakhstan (Koenig & Lennefors, 2000), the UK (Harju *et al.*, 2002; Ward *et al.*, 2007) and Iran (Mehrvar *et al.*, 2009) so far. Therefore, both RNA5 variants must be investigated separately. More details about the properties of RNA5 encoded p26 as pathogenicity factor of BNYVV P-type known so far are given in section 1.3.

1.2 Auxin signaling

Auxin is a powerful plant hormone involved in many different metabolic processes. It is involved in vascular tissue formation, tropistic responses, apical dominance, flower and fruit development but also in cellular processes, such as cell division, enlargement, differentiation (reviewed in Davies, 1995; Reed, 2001; Ori, 2019). A detailed overview of the molecular regulatory mechanisms in the auxin signaling pathway is described by Müllender *et al.*, 2021 (Manuscript I). In short, two early key elements in the regulatory auxin pathway are auxin/indole acetic acid (Aux/IAA) repressors and the interacting DNA binding auxin response

factor (ARF) activators. ARFs bind as dimers to *cis* regulatory elements, the auxin responsive elements (*AuxRE*) which leads to an activation or a repression of auxin response genes. Aux/IAA proteins themself bind as dimers to the ARFs and repress their activity as transcriptional regulators under low auxin concentrations (reviewed in Guilfoyle & Hagen, 2007; Guilfoyle, 2015; Chandler, 2016). In general, Aux/IAA proteins are short-lived, small (18-36 kDa) proteins with four highly conserved domains that are degraded at elevated auxin concentrations (reviewed in Luo *et al.*, 2018). To date, a total of 29 different Aux/IAA proteins have been identified in *Arabidopsis thaliana*, such as special Aux/IAA proteins, not sharing the typical four-part structure of the other Aux/IAA proteins. AtIAA20 (*A. thaliana* IAA20), AtIAA30, AtIAA31, AtIAA32, AtIAA33 and AtIAA34 are non-canonical Aux/IAA proteins (Sato & Yamamoto, 2008; Cao *et al.*, 2019; Lv *et al.*, 2020). These proteins lack at least one of the described domains, but often domains I and II are missing. Under high auxin conditions non-canonical are not degraded, they are stabilized by phosphorylation. This leads to a stable interaction with ARFs and the resulting regulation auf auxin responsive genes when the auxin concentration is high (Cao *et al.*, 2019; Lv *et al.*, 2020). Although the exact function of these proteins is still unknown, they are considered to be responsible for the basic adaptation of plants to different environmental conditions, as they have been found in many plant species (Jain *et al.*, 2006; Wang *et al.*, 2010; Gan *et al.*, 2013; Qiao *et al.*, 2015; Li *et al.*, 2017; Shi *et al.*, 2020). Furthermore, Aux/IAA proteins are primary auxin-responsive genes whose expression rapidly rises shortly after auxin application independently of *de novo* protein synthesis (Theologis *et al.*, 1985; Abel & Theologis, 1996; reviewed in Li *et al.*, 2016). Summing up, Aux/IAA proteins are the key regulators in the highly sensitive auxin signaling mechanism relying on degradation and synthesis of these genes controlled by the auxin level within the plant cells.

1.2.1 Lateral root formation

Root development or root embryogenesis is also controlled by auxin signaling and has been part of intensive research in recent decades. Especially in the case of sugar beet, this aspect is very interesting, as the root represents the financial economic benefit of the crop. In the following chapter, the formation and development of lateral roots (LRs) is described more detailed, as this is the economically important symptom induced by BNYVV. The development of a LR can be divided into four stages: I.) priming, II.) LR founder cell polarization, III.) LRs

initiation and IV.) patterning and LR emergence (reviewed in Lavenus *et al.*, 2013). Each of these stages is controlled by locally synthesized auxin and shoot-derived auxin. During the whole process the auxin concentration is tightly controlled by auxin transporters, such as pin-formed proteins (PIN), auxin transporter protein 1 (AUX1) or auxin transporter-like protein (LAX) (reviewed in Grones & Friml, 2015). The first stage, priming, takes place in the oscillation zone, an area of the basal meristem where periodic oscillations in auxin concentration regulate gene expression (reviewed in Moreno-Risueno *et al.*, 2010). The cells, which are affected by these oscillations are triplets of xylem pole pericycle (XPP) cell pairs, also called LR founder cells. This developmental stage functions as a pre-branch site selection process under the control of the IAA28–ARF5,6,7,8,19 auxin signaling modules that control the auxin regulated transcription factor GATA23 (De Rybel *et al.*, 2010; reviewed in Santos Teixeira & Tusscher, 2019). This is followed by the second stage, polarization of LR founder cells, an auxin-regulated stage in which the XPP cells divide anticlinally to form the LR primordium (LRP). This stage, together with the third stage, LR initiation, which is characterized by periclinal cell divisions, is controlled by solitary-root (SLR)/IAA14-ARF7,19 and bodenlos (BDL)/IAA12-MONOPTEROS(MP)/ARF5 modules. These two modules regulate the cellular processes: cell polarity/identity specification and re-entry into the cell cycle (reviewed in Lavenus *et al.*, 2013). The cell polarity/identity is accomplished via the regulated transcription factors LBD16/ASL18 and LBD29/ASL16 of the Lateral Organ Boundaries-Domain/Asymmetric Leaves2-like (LBD/ASL) family (Okushima *et al.*, 2007; Lee *et al.*, 2009). The cell cycle regulator E2Fa, the nuclear protein Aberrant Lateral root Formation 4 (ALF4), LBD18, LBD33 and the Inhibitor-Interactor of CDK/Kip Related Protein2 (ICK/KRP2) are important for re-entry into the cell cycle of the cells from the developing LR (DiDonato *et al.*, 2004; Berckmans *et al.*, 2011; Sanz *et al.*, 2011). Since the XPP cells are part of the pericycle the emerging LR has to break through three root layers, the endodermis, the cortex and the epidermis (reviewed in Péret *et al.*, 2009; Vermeer *et al.*, 2014). This is represented in the fourth step of LR formation, patterning and LR emergence. The auxin regulated module in the endodermis is called SHY2/IAA3 and the modules in the cortex and epidermis are SLR/IAA14-ARF7, 19 (Knox *et al.*, 2003; Fukaki *et al.*, 2006). Auxin, which is derived from the LRP activates these modules which leads to an elevated auxin concentration in the cells by of the auxin influx-carrier gene LIKE-AUX3 (LAX3), followed by a positive feedback loop (Swarup *et al.*, 2008). This leads to the

expression of additional transcription factors such as LBD18, which are responsible for the upregulation of cell wall remodelling proteins like pectase-lyase, subtilisin-like protease, methylesterase, β-xylosidase and expansins (*e.g.* EXP17) (Neuteboom *et al.*, 1999; Laskowski *et al.*, 2006; Lee & Kim, 2013). Due to the structural change of the surrounding cells, the LR is now able to grow through the root layers, the new LR is formed. Nevertheless, the negative regulation of LR formation is also important for the correct formation of an intact root system. This task is taken over by other hormones, such as cytokinin or ethylene, which are required as auxin antagonists leading to an inhibition of LR formation. These phytohormones interfere with auxin transport, for example, by suppressing auxin efflux carriers of the PIN family, thus preventing auxin accumulation and hence LR induction, or blocking re-entry into the cell cycle (Li *et al.*, 2006; Negi *et al.*, 2008; Lewis *et al.*, 2011). In summary, LR formation is controlled by a variety of mechanisms and processes, all of which, however, can be mainly linked to the auxin signaling pathway. Aux/IAA proteins play a central role in this process, as they mediate direct auxin responsiveness of this pathway and thus regulate transcription (reviewed in Fukaki *et al.*, 2007; Lavenus *et al.*, 2013).

1.2.2 Role of BNYVV p25 in symptom development

As described in the example of LR formation, a lot of developmental processes are tightly controlled by the auxin signaling pathway. Most of these developmental processes involve more than one signaling cascade and provide targets for viruses and other pathogens to manipulate plant development for their own advantage. A detailed overview of how different plant viruses interfere with their hosts auxin signaling pathway reviewed by Müllender *et al.* in 2021 (Manuscript I). In general, four different mechanisms have been described: 1. changing the subcellular localization of Aux/IAA proteins, 2. preventing degradation of Aux/IAA proteins by stabilization, 3. inhibiting the transcriptional activity of ARFs (reviewed in Müllender *et al.*, 2021) and recently, a fourth mechanism was discovered, 4. interaction with the SCF^{TIR1} complex (Liu *et al.*, 2021). All these interactions lead to virus-mediated transcriptional reprogramming of auxin-regulated pathways and ultimately to changes in the hosts metabolic system that are beneficial to the virus, *e.g.* suppression of plant defense, efficient virus movement and symptom development (reviewed in Müllender *et al.*, 2021).

As described in detail, auxin is essential for the regulation of root system architecture by controlling primary root elongation and lateral root (LR) formation (Muday & Haworth, 1994; Alarcón *et al.*, 2019; reviewed in Du & Scheres, 2018). Therefore, it was reasonable to assume that BNYVV interferes with the auxin signaling pathway to induce the root beard. As mentioned previously, p25 had been proposed to be the Avr determinant of the *Rz1* resistance but also to act as pathogenicity factor. This protein has been proposed to induce the root specific symptoms of BNYVV in sugar beet (Koenig *et al.*, 1991; Tamada *et al.*, 1999). Previous works already identified and characterized the interaction of p25 with BvIAA28 from *B. vulgaris* (also known as AUX28) (Thiel & Varrelmann, 2009; Gil *et al.*, 2018). It was hypothesized that this interaction is similar to the interaction of the replicase protein (Rep) from TMV with the Aux/IAA proteins AtIAA26 and AtIAA27 from *A. thaliana*. It was found, that this interaction leads to a re-localization of the Aux/IAA proteins into the cytoplasm thus inhibiting the function of the Aux/IAA proteins as transcriptional repressors (Padmanabhan *et al.*, 2005; Padmanabhan *et al.*, 2006). In the case of TMV this alteration of the subcellular localization of Aux/IAA proteins, leads to activation of auxin signaling because Aux/IAA proteins can no longer exert their suppressive effect on ARFs whereas the other interactions lead to suppression of auxin signaling. Such a “shuttling function” has been also identified for p25 which encodes a nuclear localization signal (NLS) and a nuclear export signal (NES) (Vetter *et al.*, 2004). Using fluorescent localization markers, it has been shown that the BvIAA28, which is actually strictly restricted to the nucleus, can also be detected in the cytoplasm when co-expressed with p25, suggesting re-localization (Gil *et al.*, 2018). On the basis of this, it was hypothesized that p25 enters the nucleus via an NLS signal during pathogenesis and exports interacting Aux/IAA proteins via the NES signal, thereby downregulating their function and causing the root beard formation (Gil *et al.*, 2018). Furthermore, a detailed characterization of this interaction revealed that p25 specifically interacts with domain I and II via a domain mapping of BvIAA28 (Gil *et al.*, 2018). These domains are responsible for repressive activity and auxin responsiveness of the Aux/IAA proteins (Szemenyei *et al.*, 2008; Song & Xu, 2013; reviewed in Müllender *et al.*, 2021). This further clarifies, that p25 seems to repress the activity of BvIAA28.

1.3 RNA5 encoded p26 as pathogenicity factor of BNYVV P-type

As mentioned above, the P-type carries the additional genomic component RNA5, encoding the 26 kDa protein p26 (Tamada *et al.*, 1989). Since no reverse genetic system for the P-type has been available so far, this pathotype could only be studied in field populations or by means of reassortants with the A-type cDNA clone supplemented with P-type RNA5. (Heijbroek *et al.*, 1999; Bornemann & Varrelmann, 2011; Liebe *et al.*, 2020; Tamada *et al.*, 2020). Bioassays under greenhouse conditions with BNYVV field populations of the A-, B- and P-type revealed, that the P-type is more pathogenic and causes more severe foliar symptoms than the other types (Heijbroek *et al.*, 1999). Tamada and coworkers further described a more severe LR proliferation as well as scab-like symptoms caused by RNA5 when inoculated with naturally infested field soil (Tamada *et al.*, 2020). Furthermore, they investigated the difference in virus-induced sugar yield losses between Japanese BNYVV isolates with and without RNA5. RNA5-containig isolates caused a sugar reduction of 39% whereas the sugar reduction of BNYVV isolates without RNA5 was only 25% relative to control plants (Tamada *et al.*, 2020). This was attributed to the higher accumulation of viral RNA3 in isolates containing RNA5. It appears that both proteins act as pathogenicity factors and affect the expression of root symptoms maybe as consequence of an interference of both proteins. Liebe and colleagues demonstrated in 2020 that only one of the two proteins is required for successful viral replication. They proved that RNA3 can be replaced by P-type RNA5 in the infectious A-type clone. This can be explained by the fact that p25 and p26 might be derived from a common ancestral protein since they exhibit quite strong sequence similarities (e-value: 4 × 10−10, 22% sequence identity, and a 43% positive match in a 217 amino acid region) (Simon-Loriere & Holmes, 2013).

As described above, resistance-breaking of BNYVV A-type isolates has been associated with variation of the hypervariable tetrad of p25 (Koenig *et al.*, 2009b; Acosta-Leal *et al.*, 2010; Liebe *et al.*, 2020). The P-type has also been shown to break *Rz1* resistance (Pferdmenges *et al.*, 2008; Bornemann & Varrelmann, 2013), but independently of RNA3. It has been shown, that P-type RNA5 has the ability to mediate *Rz1* resistance-breaking in an A-type background even without RNA3 (Liebe *et al.*, 2020). This supports the idea of a second pathogenicity factor and the evolution of two independent resistance-breaking strategies, as proposed by Tamada *et al*. in 2020. So far, the mechanism how RNA5 contributes to *Rz1* resistance-breaking

remains unclear. One molecular analysis, revealed that p26 is partially targeted to the nuclear compartment of infected *C. quinoa* cells by means of transient expression of RNA5 (Link *et al.*, 2005). Furthermore is has been shown, that p26 strongly activates transcription in a yeast one-hybrid system (Link *et al.*, 2005; Covelli *et al.*, 2009). Whether and how these observations are related to the pathogenicity of the P-type remains to be clarified, but first clear and strong evidence must be brought that RNA5 also has symptom enhancing effects in the P-type background. To confirm this, a reverse genetic system with all five RNA components of the P-type must be generated. Furthermore, such a system would allow to make more reliable statements about the resistance-breaking properties of the P-type.

2. Research objectives

BNYVV is the most important viral disease in sugar beet cultivation, as infested areas can have a sugar yield loss of up to 80%. These losses are mainly caused by the characteristic root beard, a massive LR proliferation of infected sugar beets, leading to tap-root size reductions. Since the development of LRs is mainly controlled by auxin, it is reasonable to assume that BNYVV interferes with the Auxin signaling pathway. Additionally, past studies have already found preliminary evidence that the root beard is induced by an interaction of the virus with the auxin signaling pathway from sugar beet. Based on various studies, it was also found that symptom expression differs between the BNYVV pathotypes. The BNYVV P-type is assumed to cause more severe symptoms than the A- or B-type. Furthermore, there is evidence that the P-type can overcome *Rz1* but not *Rz2* resistance.

Before starting the experimental work, a comprehensive literature research on other viruses interacting with the auxin signaling pathway of their respective host was done. Some viruses are known to interfere with the signaling pathway at various points, thus affecting plant development such as tobacco mosaic virus (TMV), rice dwarf virus (RDV), southern rice lack streaked dwarf virus (SRBSDV), rice black streaked dwarf virus (RBSDV), rice stripe virus (RSV) and rice stripe mosaic virus (RSMV). BNYVV is also assumed to interfere with the auxin signaling pathway, as auxin mainly controls LR formation. Preliminary indications suggested that this might be due to an interaction of the viral pathogenicity factor p25 with sugar beet Aux/IAA proteins. This research was done to get an idea of how other plant viruses can interfere with the auxin signaling and to find possible approaches for analyses and experiments, as well as to make initial hypotheses on how exactly BNYVV might interact with the auxin signaling pathway (manuscript I).

The main part of this thesis, however, was to further confirm and characterize the interaction of the viral pathogenicity factor p25 from BNYVV with the sugar beet auxin signaling pathway. At first, investigations on the interaction with the auxin signaling pathway and a change of the auxin content in BNYVV infected lateral roots were conducted. The interaction of BvIAA28 with p25 has already been identified and characterized in other studies, however, there were 12 additional BvIAA proteins to be tested for interaction with p25. For further characterization, interacting domains of the partners as well as the subcellular localization of

the Aux/IAA proteins and p25 were aimed to be identified. Since gene silencing or overexpression is not yet possible in sugar beet, the Aux/IAA proteins interacting with p25 were heterologously expressed in *N. benthamiana* plants to investigate phenotypical changes. This provided new insights into how exactly p25 disrupts auxin signaling and triggers root beard (manuscript II).

In addition, a cDNA clone of the P-type was created to analyze this pathotype under controlled greenhouse conditions. First, phenotypic differences of leaves and roots between the P- and A-type infected sugar beet plants were presented. In addition, to investigate the resistance-breaking properties of the P-type, *Rz1* and *Rz2* resistant sugar beet varieties were inoculated with the cDNA clone of the P-type and tested for virus replication. Finally, it was tested whether RNA5 (p26) is responsible for resistance-breaking of *Rz1*. For this purpose, the P-type was tested for resistance-breaking properties with and without RNA5. Finally, the evolutionary relationships of the P-type with the A-type was investigated by reassortant experiments as well as *in silico* studies (manuscript III).

3. Publications

Manuscript I

Manipulation of auxin signalling by plant viruses

Maximilian Müllender[1], Mark Varrelmann[1], Eugene I. Savenkov[2], Sebastian Liebe[1*]

[1]Institute of Sugar Beet Research, Department of Phytopathology, Göttingen, Germany

[2]Department of Plant Biology, Uppsala BioCenter SLU, Swedish University of Agricultural Sciences, Linnean Center for Plant Biology, Uppsala, Sweden

***Correspondence:** Liebe, Sebastian <liebe@ifz-goettingen.de>

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Abstract

The compatible plant-virus interactions result in dramatic changes of the plant transcriptome and morphogenesis, and are often associated with rapid alterations in plant hormone homeostasis and signalling. Auxin controls many aspects of the plant organogenesis, development and growth, therefore, plants can rapidly perceive and respond to the changes in the cellular auxin levels. The auxin signalling is a tightly controlled process and, hence, is highly vulnerable to changes in the mRNA and protein levels of its components. There are several core nuclear components of auxin signalling. In the nucleus, the interaction of auxin response factors (ARF) and auxin/indole acetic acid (Aux/IAA) proteins is essential for the control of auxin-regulated pathways. Aux/IAA proteins are negative regulators whereas ARFs are positive regulators of the auxin-response. The interplay between both is essential for the transcriptional regulation of auxin-responsive genes which primarily regulate developmental processes, but also modulate the plant immune system. Recent studies suggest that plant viruses belonging to different families have developed various strategies to disrupt auxin signalling, namely by (i) changing the subcellular localisation of Aux/IAAs, (ii) preventing degradation of Aux/IAAs by stabilisation or (iii) inhibiting the transcriptional activity of ARFs. These interactions perturb auxin signalling and experimental evidence from various studies highlight their importance for virus replication, systemic movement, interaction with vectors for efficient transmission and symptom development. In this microreview, we summarize and discuss the current knowledge on the interaction of plant viruses with auxin signalling components of their hosts.

Introduction

Plant viruses are of great importance to agriculture as they constantly threaten crop production by causing major economic losses in yield and quality of harvested tissue (Rybicki, 2015; Scholthof et al., 2011). The interaction of viruses with their host plants is often associated with rapid alterations in phytohormone homeostasis and signalling which is an important aspect in plant–virus interactions as highlighted in a recent review (Zhao & Li, 2021). Plant defense hormones, namely, salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are important for mounting the primary defense responses to the pathogen attack, whereas growth-related phytohormones including auxin, cytokinins, brassinosteroids, abscisic acid and

gibberellins can modulate the plant immune system (Han and Kahmann, 2019; Islam et al., 2019). Auxin controls a multitude of cellular and developmental processes including cell division and enlargement, differentiation, vascular tissue formation, tropic responses to light and gravity, apical dominance and organ development (Abas et al., 2006; Dharmasiri et al., 2005b; Friml et al., 2002; Gray et al., 2001; Ori, 2019). The major natural auxin occurring in plants is indole-3-acetic acid (IAA), and plants have universally conserved machinery for its synthesis.

Alterations in the host auxin metabolism are important for plant-microbe interactions as these changes stimulate plant cell growth, modulate defense responses and alter host physiology. Bacterial and fungal plant pathogens can interfere with the auxin metabolism by pathogen-produced enzymes which either synthesize or inactivate auxin (Kunkel and Harper, 2018; Ludwig-Müller, 2015). Plant viruses do not encode such enzymes owning to the limitations imposed by the small size of their genomes. The small genome size of plant viruses means that most viral proteins are multifunctional suggesting that some viral proteins might subvert phytohormone-mediated responses (e.g. through direct interaction with signalling components) for the virus benefit. Indeed, research over the last decade, mostly on RNA viruses, has established that plant viruses are able to manipulate auxin signalling of their hosts for their own advantage.

Mechanism of auxin sensing in plants

Auxin response factors (ARF) and auxin/indole acetic acid (Aux/IAA) proteins are key components in the regulation of auxin signalling events. Members of the ARF transcription factor (TF) family across plant species share four highly conserved domains. ARFs bind as dimers to auxin responsive elements (AuxRE) in the promotors of auxin-regulated genes via an N-terminal B3-type DNA binding domain (DBD). The variable middle region of ARF proteins functions as either activation or repression domain for auxin-responsive genes. The carboxyl-terminal dimerization domain (CTD) contains a Phox/Bem1p domain (PB1) which mediates homo- and heterodimerization, as well as heterodimerization with Aux/IAA proteins under low auxin concentrations (Figure 1A) (Chandler, 2016; Guilfoyle, 2015; Guilfoyle and Hagen, 2007; Piya et al., 2014). Aux/IAA proteins represent key regulators in the auxin-mediated signalling as they are able to respond to the auxin levels in the cells.

Aux/IAAs are short-living, small (18 – 36 kDa) proteins with four highly conserved domains (Abel and Theologis, 1996; Oeller et al., 1993). The N-terminal domain I (DI) is characterised by the presence of the consensus sequence LxLxL (where L refers to leucine amino acid residue and x to any amino acid residue), a conserved ethylene response factor-associated amphiphilic repression (EAR) motif (Tiwari et al., 2004). At low auxin concentrations, this domain is responsible for the dominant repressive activity of Aux/IAA proteins as it binds to tetramers of the co-repressors TOPLESS (TPL) and TOPLESS-RELATED (TPR) (Szemenyei et al., 2008). TPL/TPR co-repressors harbour WD40 repeats, which recruit chromatin modifying enzymes such as histone deacetylases (HDACs). HDACs modify chromatin to be transcriptionally inactive, leading to repression of auxin-responsive genes (Causier et al., 2012; Ke et al., 2015; Kieffer et al., 2006). Domain II (DII) contains the primary degron sequence qv**VGWPP**vrsy**R**k**N** (highly conservative residues are in bold and underlined) that mediates the auxin responsiveness (Song and Xu, 2013). The C-terminal domains III and IV (DIII, DIV) of Aux/IAAs are similar to the Phox/Bem1p domains of ARFs that allow interactions among these TFs and, hence, suppress the regulatory activities of ARFs (Dinesh et al., 2015; Guilfoyle, 2015; Guilfoyle and Hagen, 2012; Korasick et al., 2015; Tiwari et al., 2004).

When the auxin concentration increases (Figure 1B), Aux/IAA proteins are ubiquitinated by a ubiquitin SCF-type E3 ligases (E3) via an E1/E2 enzyme system and degraded by the 26S proteasome (Dharmasiri et al., 2005a; Hershko, 1998; Leyser, 2018; Pickart, 2001; Tan et al., 2007; Thelander et al., 2019). Auxin acts as a molecular glue and connects Leu-rich repeats of F-box proteins with the conserved degron motif (DII) of Aux/IAAs (Tan et al., 2007). As part of the SCF-type E3 ligases, the F-box protein conveys the substrate specificity to the Aux/IAAs (Hayashi et al., 2008; Ruegger et al., 1998). SCF-type E3 ligases are named after their three subunits: S-PHASE KINASE-ASSOCIATED PROTEIN 1 (SKP1), a RING-box protein 1 (RBX1), CULLIN 1 (CUL1) dimer and the F-box protein (TIR1 F-box) (Deshaies, 1999). The F-box is part of the auxin perceiving co-receptor family TRANSPORT INHIBITOR RESPONSE 1 / AUXIN SIGNALLING F-BOX 1-5 (TIR1/AFB) (Kepinski and Leyser, 2005; Tan et al., 2007). The RBX1 CUL1 dimer catalyses ubiquitin polymerisation and is responsible for ubiquitination of the target proteins. The multiprotein complex responsible for the auxin-dependent interaction and subsequent degradation of Aux/IAAs is called SCF^{TIR1} (Dharmasiri et al., 2005b; Prigge et al., 2016; Ruegger et al., 1998).

Upon degradation of Aux/IAAs, ARFs can act as TFs regulating the expression of primary auxin-responsive genes. Three gene families including *Small Auxin Up-regulated RNA* (*SAUR*), *Gretchen Hagen 3* (*GH3*) and *Lateral Organ Boundaries Domain* (*LBD*) are often a part of an early auxin-response (Catalá et al., 2000; Fan et al., 2012; Hagen and Guilfoyle, 1985; Knauss et al., 2003). *Aux/IAAs* are also primary auxin responsive genes, whose expression is rapidly elevated shortly after auxin application (Abel and Theologis, 1996; Li et al., 2016; Theologis et al., 1985).

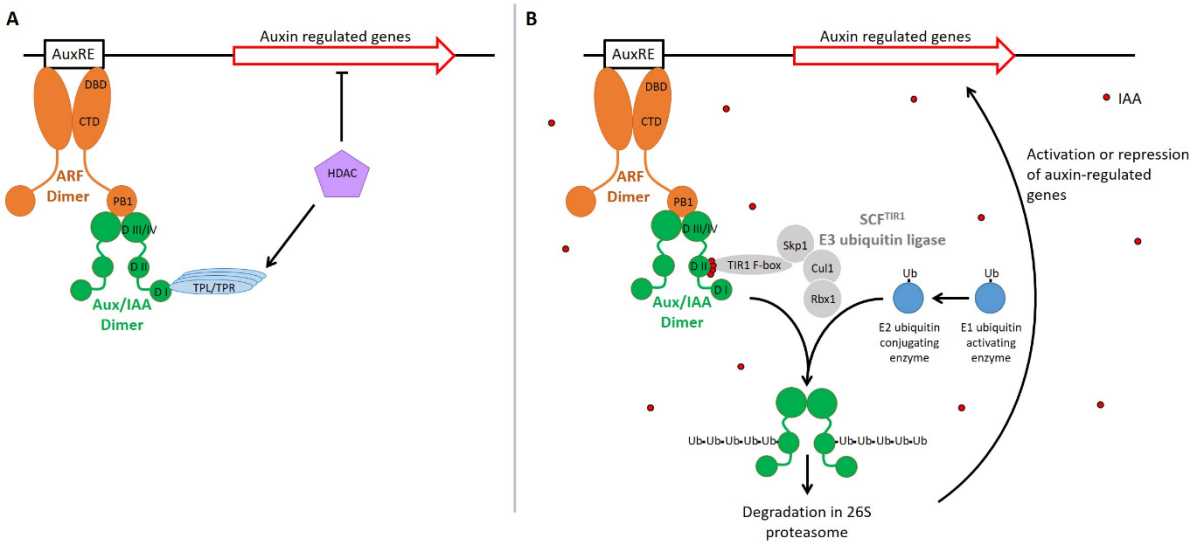

Figure 1. (A) State of the auxin signalling pathway under low auxin conditions. Auxin response factors (ARF) are bound as dimers (CTD - carboxyl-terminal dimerization domain) to auxin responsive elements on the DNA (AuxRE) with their B3-type DNA binding domain. Aux/IAA dimers are bound via their domain II/IV (D III/IV) to a type I/II Phox and Bem1p (PB1) protein–protein interaction domain. With domain I (D I) AUX/IAAs interact with TOPLESS and TOPLESS-RELATED co-repressors (TPL/TPR) which recruit a histone deacetylase (HDAC). Resulting modifications of the DNA lead to a downregulation of the transcriptional activity of auxin regulated genes. **(B)** State of the auxin signalling pathway under high auxin conditions. Auxin acts as molecular glue between domain II of Aux/IAA proteins and the SCFTIR1 E3 ubiquitin ligase complex (Skp1 - subunits S-phase kinase-associated protein 1, Rbx1 - RING-box protein 1, Cul1 - Cullin 1, TIR1 F-box - F-box protein). Ubiquitin (Ub) is first activated by the E1 ubiquitin activating enzyme and then bound to domain II of Aux/IAA proteins via an E2 ubiquitin conjugating enzyme and the Rbx1 subunit of the SCFTIR1 E3 ubiquitin ligase. The ubiquitinated Aux/IAA proteins are degraded in the 26S proteasomes and are no longer bound to ARF dimers. ARF dimers are released and can now operate as transcriptional activators or repressors.

Plant-virus infections induce changes in auxin metabolism

Auxin metabolism comprises biosynthesis, conjugation, and degradation (Casanova-Sáez et al., 2021). It is now well established that IAA is mainly synthesised from tryptophan via indole-3-pyruvic acid (IPyA) pathway (Chen et al., 2020; Woodward, 2005; Zhao, 2001; Zheng et al., 2013), whereas several other redundant pathways function in parallel including auxin

production via tryptamine (TRA) (Pollmann et al., 2002; Pollmann et al., 2003; Hull et al., 2000; Mikkelsen et al., 2000; Facchini et al., 2000). The inactivation of auxin is important to maintain auxin homeostasis in plants (Ljung, 2013). Metabolic inactivation of IAA is performed through oxidation and conjugation processes. Whereas auxin-inducible acyl amino synthetases of the *GH3* gene family convert IAA to IAA-amino acid conjugates (Staswick et al., 2005), uridine diphosphate glucosyltransferase oxidizes IAA into 2-oxindole-3-acetic acid (Peer et al., 2013; Pěnčík et al., 2013).

Viral infections are often accompanied by changes in the expression of the key genes of these pathways leading to either increase in accumulation or decrease in cellular levels of auxin. In rice plants infected with *Rice black streaked dwarf virus* (RBSDV; Genus: *Fijivirus*; Family: *Reoviridae*), the concentration of the main active form of IAA gradually decreases whereas the amount of the intermediate degradation product, IAA-aspartate, sharply increases (Huang et al., 2018). This coincides with down-regulation of auxin-biosynthesis genes and a strong up-regulation of the *GH3.8* gene encoding an IAA-amino synthetase responsible for the synthesis of IAA-aspartate conjugate (Zhang et al., 2019). In contrast, sugar beet plants infected with *Beet necrotic yellow vein virus* (BNYVV; Genus: *Benyvirus*; Family: *Benyviridae*) are characterised by elevated auxin levels (Pollini et al., 1990). Furthermore, in those plants, the *GH3.1* gene, involved in auxin conjugation and inactivation, is strongly down-regulated (Gil et al., 2020). Similarly, *Rice dwarf virus* (RDV; Genus: *Phytoreovirus*; Family: *Reoviridae*) triggers auxin biosynthesis in rice (Qin et al., 2020).

In *A*. *thaliana*, the expression of HC-Pro, a viral suppressor of RNA silencing (VSR) of *Tobacco vein banding mosaic virus* (TVBMV; Genus: *Potyvirus*; Family: *Potyviridae*), decreases the DNA methylation in the promotors of the *YUCCA* genes of the IPyA pathway leading to transcriptional activation of these genes, and ultimately, to elevated auxin levels (Yang et al., 2020). Moreover, transcriptional changes in auxin-responsive genes have been also reported for many other plant-virus pathosystems (Li et al., 2017; Liu et al., 2019; Padmanabhan et al., 2019; Pierce and Rey, 2013; Zhou et al., 2016), and therefore seem to be a general response of plants to virus infection.

Plant viruses disrupt auxin sensing by targeting Aux/IAA proteins: The case studies

Tobacco mosaic virus

The interaction between a viral protein and a plant Aux/IAA was first described for *A. thaliana* - *Tobacco mosaic virus* pathosystem (TMV; Genus: *Tobamovirus*; Family: *Virgaviridae*) (Padmanabhan et al., 2005). IAA26 was found to interact with the helicase domain of the TMV replicase (Figure 2A). The nuclear localisation of IAA26 was disrupted by coexpression with the TMV replicase leading to a cytoplasmatic distribution of IAA26. Therefore, it was hypothesised that translocation of IAA26 to the cytoplasm impairs its putative function as a transcriptional regulator of auxin-responsive genes in the nucleus (Padmanabhan et al., 2005; Padmanabhan et al., 2006). Indeed, this hypothesis was supported by changes in the transcript levels of auxin-responsive genes in TMV infected plants. Furthermore, transgenic plants silenced for *IAA26* showed TMV like symptoms. Additionally, a TMV mutant (TMV-V1087I) expressing an altered replicase with a single amino acid substitution (V1087I) was incapable of interacting with IAA26. This did not lead to a change of the subcellular localisation of IAA26 and induced only attenuated developmental symptoms in the infected plants. The TMV-V1087I mutant replicated and spread in young leaf tissue similar to the wild type (wt) virus, but the virus accumulation was reduced in older tissue (Padmanabhan et al., 2008). The protein levels of IAA26 were found to be higher in mature tissue, and therefore it was concluded that the interaction of TMV replicase with IAA26 is crucial for supporting virus replication in older leaves. Consequently, the accumulation of the TMV-V1087I mutant was further reduced in transgenic *A*. *thaliana* plants expressing a degradation resistant variant of IAA26 (Padmanabhan et al., 2008). Later, it was shown that IAA26 is predominantly expressed in the vascular tissue and its nuclear localisation is disrupted by TMV in companion cells of the vascular bundle (Collum et al., 2016). The ability of wt TMV to interact with Aux/IAAs resulted in an increased ability for phloem loading and systemic spread in mature tissue compared to the mutant TMV-V1087I.

Interestingly, the expression levels of *pectin methylesterase 5* (*PME5*), *microtubule end-binding 1a* (*EB1a*), *PD-located protein 3* (*PDLP3*) and members of the *β-1,3-glucanase* gene family were altered in transgenic plants overexpressing a degradation resistant IAA26 variant (Collum et al., 2016). It is assumed that these genes are involved in cell-to-cell movement of

TMV. Additionally, the expression levels of defense related genes were changed suggesting that the interaction of TMV with IAA26 is also important for mounting an antiviral defense. The interaction of TMV with IAA26 seems to be mediated by a highly conserved domain of IAA26 because the orthologue proteins from tomato and *Nicotiana benthamiana* also interact with TMV replicase leading to a disruption of their nuclear localisation (Collum et al., 2016; Padmanabhan et al., 2008). Knock-down of the *IAA26* expression in tomato resulted in a phenotype similar to TMV infected plants. Besides IAA26, two other *A*. *thaliana* Aux/IAA proteins, namely IAA27 and IAA18, were found to interact with TMV replicase, but with lower affinity as compared to IAA26 (Padmanabhan et al., 2006). Furthermore, upon TMV infection, only the nuclear localisation of IAA27 was disrupted whereas the localisation of IAA18 to the nucleus was not affected. So far, the role of IAA27 and IAA18 in TMV pathogenesis remains elusive.

Rice dwarf virus

The mechanism by which plant viruses manipulate auxin signalling has been also well characterised for RDV causing dwarfism in rice. Genes involved in early synthesis of IAA as well as auxin-responsive genes are down-regulated during RDV infection (Satoh et al., 2011). The RDV P2 protein interacts with domain II of OsIAA10, which impedes the interaction of OsIAA10 with OsTIR1 (Jin et al., 2016) (Figure 2B). Moreover, OsIAA10 is stabilised by P2 in a dose-dependent manner and its degradation through auxin perception by the $SCF^{TIR1/AFBs}$ complex is prevented. Transgenic rice plants overexpressing *OsIAA10* develop an auxin-resistant phenotype that resembles symptoms of RDV-infected rice plants including stunting, higher number of tillers, shorter crown roots and lower seed fertility. Moreover, these transgenic plants display more severe symptoms after natural RDV infection whereas knock-out of the *OsIAA10* expression reduce virus replication and symptom severity. These findings highlight the important role of the interaction between P2 and OsIAA10 for enhancing virus infection.

The active role of auxin in the defense against RDV was addressed in a recent study (Qin et al., 2020). Two ARF proteins, namely, OsARF12 and OsARF16, were identified as interaction partners of OsIAA10, which positively regulates rice antiviral defense against RDV. Moreover, OsWRKY13 TF was identified as a target of OsARF12 as OsARF12 binds to an AuxRE element in

the promoter of *OsWRKY13* to activate transcription of the gene. Knockout of *OsWRKY13* increases virus accumulation and symptom severity. Consequently, the increase of auxin content in RDV-infected rice plants leading to degradation of OsIAA10 and transcription activation of OsWRKY13 by OsARF12 appears to be a part of an auxin-mediated defense response against RDV (Qin et al., 2020). However, RDV has developed a counter-defense strategy by stabilising OsIAA10 that leads to repression OsARF12 and OsARF16 and dampening OsARF12- and OsARF16-mediated anti-viral responses (Jin et al., 2016). Interestingly, P2 is targeted for degradation by the rice E3 ubiquitin ligase OsRFPH2-10 as part of an antiviral defense at the early stages of infection (Liu et al., 2014).

Besides the auxin signalling pathway, RDV can hijack signalling pathways of other phytohormones to enhance infection and virus multiplication. P2 interacts with *ent*-kaurene oxidases leading to reduced accumulation of GA, which, in turn, results in a dwarf phenotype of RDV-infected rice plants (Zhu et al., 2005). Furthermore, the RDV-encoded protein Pns11 interacts with OsSAMS1 and enhances its enzymatic activity leading to higher ethylene levels, which in turn result in enhanced severity of the virus symptoms in RDV-infected rice plants (Zhao et al., 2017). Thus, collectively the disease symptoms induced by RDV are probably the result of disrupting signalling pathways of several phytohormones.

Beet necrotic yellow vein virus

Another plant virus known to interfere with auxin signalling pathways is the BNYVV causing rhizomania disease in sugar beet. The taproot of BNYVV-infected sugar beet plants is characterised by massive lateral root (LR) formation which requires the presence of the P25 virulence factor (Tamada et al., 1999). LR formation is a developmental process governed by auxin and specific Aux/IAA-ARF-modules (Trinh et al., 2018). The taproot of infected sugar beet plants undergoes comprehensive transcriptional reprogramming of auxin regulated pathways (Gil et al., 2018; Gil et al., 2020; Schmidlin et al., 2008). This includes in particular the up-regulation of *LBD* TFs and *EXPANSINSs* (EXPs), both of which are crucial for LR development. *LBD* TFs are directly activated by ARFs and can activate the expression of *EXP* genes (Lee et al., 2013; Lee and Kim, 2013; Okushima et al., 2007), which encode cell wall loosening proteins needed for cell elongation during LR formation (Cosgrove, 2015). Additionally, genes involved in auxin biosynthesis via the IPyA and TRA pathways are also

strongly activated during BNYVV infection (Gil et al., 2020), which is in accordance with the observation of higher auxin levels in BNYVV infected taproots (Pollini et al., 1990). However, recently, elevated levels of the conjugated inactive form of auxin (IAA-Ala) were detected in BNYVV-infected sugar beet plants suggesting a compensatory plant response to maintain auxin homeostasis (Webb et al., 2020).

A sugar beet cDNA library was screened using yeast two-hybrid to identify host proteins that interact with the P25 virulence factor (Thiel and Varrelmann, 2009). The screen yielded IAA28 as a P25 interacting partner (Gil et al., 2018; Thiel and Varrelmann, 2009). IAA28-P25 interaction occurs via IAA28 domains I and II (Gil et al., 2018). Subcellular localisation of co-expressed P25 and IAA28 revealed that P25 inhibits IAA28 nuclear localisation similar to the TMV case described above (Figure 2A). Interestingly, BNYVV infected sugar beet plants characterised by massive LR formation resemble the appearance of the tomato plants silenced for Aux/IAA genes (Bassa et al., 2012). By contrast, suppression of LR formation and extreme stunting of the plants is a typical phenotype of the Aux/IAA-over expressing lines of *A*. *thaliana* (Fukaki et al., 2002; Rogg et al., 2001). Thus, P25 presumably inactivates the transcriptional repressor activity of IAA28 through the disruption of its nuclear localisation, again, a mechanism seems to be similar to the interaction of TMV with auxin signalling as described above. Alternatively, P25 may trigger a 26S proteasome mediated degradation of IAA28, but this hypothesis needs to be addressed in future experiments.

The interaction of the P25 virulence factor with auxin signalling pathways seems to be occurring via signalling components sharing some level of conservation between sugar beet (host of BNYVV) and *A. thaliana* (a non-host for BNYVV) as transgenic *A. thaliana* plants expressing P25 are characterised by increased auxin content, abnormal root branching phenotype, and differential expression of auxin responsive genes (Peltier et al., 2011). Additionally, these transgenic *A*. *thaliana* plants are more susceptible to a treatment with the synthetic auxin 2,4-D, supporting the idea that P25 increases auxin sensitivity by disrupting the transcriptional activity of AUX/IAA proteins via yet unknown mechanism. In contrast to sugar beet (*Beta vulgaris subsp. vulgaris*) and *A. thaliana*, the experimental host *N. benthamiana* and the crop wild relative subspecies *Beta vulgaris subsp. macrocarpa* display stunting, leaf curling and root developmental defects after BNYVV infection. These symptoms resemble an auxin-insensitive phenotype suggesting that in these particular species P25 might

stabilize IAA28 (or/and other AUX/IAA proteins) similar to RDV P2-IAA10 interactions described above. These questions require further investigation. However, additional alternatives deserve consideration as small RNA-seq and subsequent validation of the data revealed an up-regulation of miR396 (in both species in question) resulting in down-regulation of the TIR1 auxin receptor transcript, the cleavage target of miR396 (Fan et al., 2015; Liu et al., 2020). The repression of the auxin response by reducing the expression of the auxin receptor may indicate a host specific effect of BNYVV on the auxin signalling pathway in the host other than sugar beet.

Plant viruses disrupt transcriptional activity of ARFs

Besides interaction with Aux/IAA proteins, plant viruses are also able to target ARF TFs and disrupt their transcriptional activity (Figure 2C). In a comprehensive study (Zhang et al., 2020) investigated the interaction of the rice infecting viruses *Southern rice black streaked dwarf virus* (SRBSDV; Genus: *Fijivirus*; Family: *Reoviridae*), *Rice black streaked dwarf virus* (RBSDV; Genus: *Fijivirus*; Family: *Reoviridae*), *Rice stripe virus* (RSV; Genus: *Tenuivirus*; Family: *Phenuiviridae*) and *Rice stripe mosaic virus* (RSMV; Genus: *Cytorhabdovirus*; Family: *Rhabdoviridae*) with ARFs. The two related proteins SP8 from SRBSDV and P8 from RBSDV were found to specifically interact with the CTD domain of OsARF17 preventing its dimerization and leading to a suppression of its activity as a TF. Furthermore, overexpression of OsARF17 reduced accumulation of both viruses whereas virus accumulation and symptom severity were enhanced in the knockout mutant rice lines. In the same study the P2 protein of the distantly related RSV was found to interact with the DBD domain of OsARF17 that impeded its interaction with AuxREs in the promoters and, therefore, the transcription activation of auxin response genes. Similar to SRBSDV and RBSDV, the accumulation of RSV and symptom severity were reduced in the transgenic rice lines overexpressing OsARF17. Finally, the authors showed that the M protein from the *Cytorhabdovirus* RSMV interacts with the MR-CTD domain of OsARF17 and represses its transcriptional activity. Overexpression of OsARF17 resulted in reduced virus accumulation similar to the aforementioned viruses. Thus, OsARF17 is important for antiviral defense in rice and several plant viruses have independently evolved strategies aiming at disrupting the transcriptional activity of this protein.

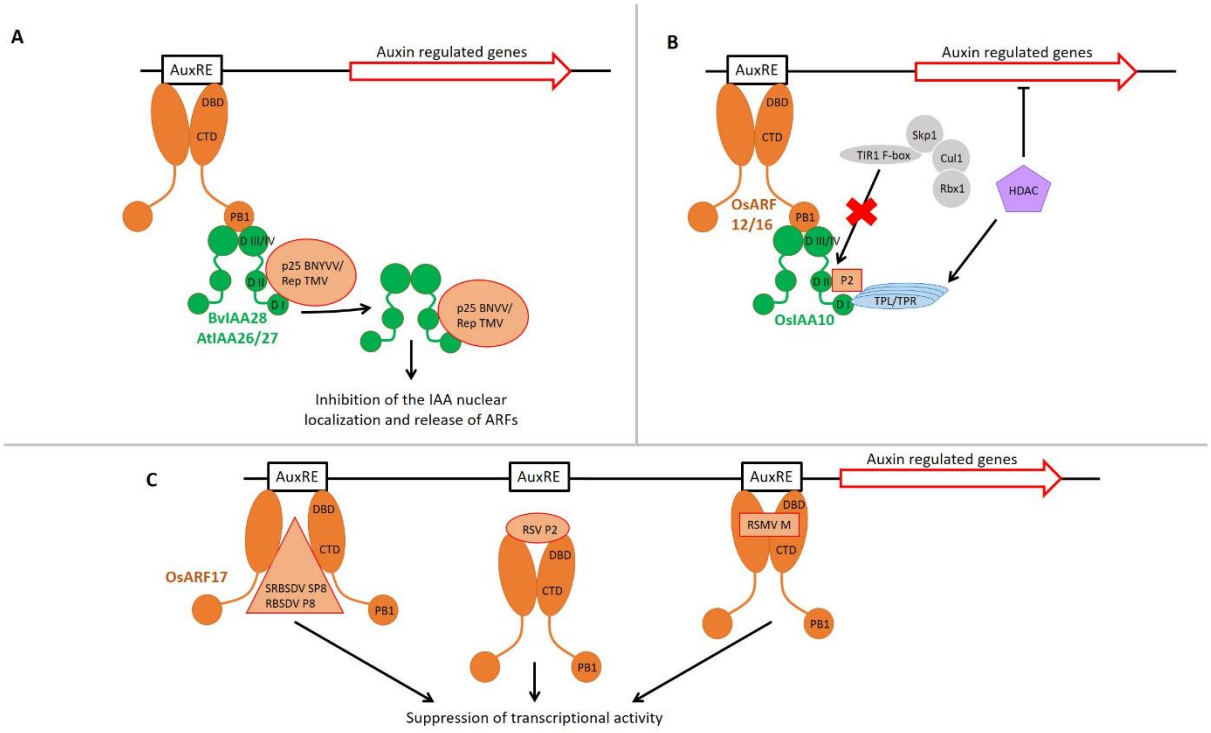

Figure 2. (A) Interaction of the pathogenicity factors from the Beet necrotic yellow vein virus (BNYVV) – p25 and from Tobacco mosaic virus (TMV) – replicase (Rep) with the Aux/IAA proteins BvIAA28 or AtIAA26/27 from sugar beet, *Beta vulgaris* of *Arabidopsis thaliana* respectively. This interaction inhibits the nuclear localisation of Aux/IAAs and suppresses their regulatory properties, leading to the release of the ARFs which can then take over their role as transcription factors again. **(B)** Interaction of *Rice dwarf virus* (RDV) with the auxin signalling pathway of rice, *Oryza sativa*. OsIAA10 is stabilised by the viral protein P2 in a dose-dependent manner. An interaction of the $SCF^{TIR1/AFBs}$ complex with domain II (D II) of OsIAA10 is prevented by P2 even under high auxin concentration. OsARF12 and 16 are still suppressed by OsIAA10 and genes involved in early synthesis of IAA as well as auxin responding genes are down-regulated during infection. **(C)** Interaction of the rice infecting viruses Southern rice black streaked dwarf virus (SRBSDV, SP8), Rice black streaked dwarf virus (RBSDV, P8), Rice stripe virus (RSV, P2) and Rice stripe mosaic virus (RSMV, M protein) with ARFs. SP8 from SRBSDV and P8 from RBSDV were found to specifically interact with the CTD domain of OsARF17 preventing its dimerization. P2 protein of the RSV was found to interact with the DBD domain of OsARF17 which impeded the interaction with AuxREs and the M protein from RSMV interacts with the MR-CTD domain of OsARF17. All these interactions lead to a suppression of transcriptional activity of OsARF17.

Conclusion

As described above, plant viruses have developed diverse strategies to disrupt auxin signalling either by (i) changing the subcellular localisation of Aux/IAAs, (ii) preventing degradation of Aux/IAAs by stabilisation or (iii) inhibiting the transcriptional activity of ARFs. This leads to either activation (i) or suppression (ii and iii) of the auxin signalling. Overall, these changes result in virus-mediated transcriptional reprogramming of auxin-regulated pathways which ultimately can lead to a suppression of plant defense, efficient virus movement and symptom development. As shown for TMV, the interaction with Aux/IAAs can help viruses to replicate

and move better in older leaf tissue where Aux/IAAs are present in higher levels. Thus it is speculated that disruption of auxin signalling reprogram older cells to make them more compatible for virus replication and movement (Padmanabhan et al., 2008). Whether the disruption of auxin signalling also activates a negative or positive feedback loop leading to suppression or activation of auxin biosynthesis remains unclear.

The effects of virus infections on the expression of genes involved in auxin metabolism and the alteration of cellular auxin levels cannot be separated from the host responses. Plants constantly have to adjust catabolic and anabolic auxin pathways acting together with auxin carriers to regulate cellular auxin homeostasis and to respond to developmental and environmental cues (Rosquete et al., 2012). Furthermore, auxin is also in a close cross-talk with stress related hormones including SA, JA and ET which collectively also affect its homeostasis (Naseem et al., 2015; Robert-Seilaniantz et al., 2011; Yang et al., 2019). The defense-related phytohormone SA represses the auxin signalling (Wang et al., 2007; Yuan et al., 2017), whereas the JA signalling can induce auxin synthesis (Hentrich et al., 2013).

Auxin is also of a similar importance in bacterial and fungal host-interactions. For example, *Botrytis cinerea* and *Pseudomonas syringae* induce the accumulation of the conjugated form IAA-Asp in *A*. *thaliana* that enhances disease development due to inactivation of auxin (González-Lamothe et al., 2012). In contrast, *Fusarium oxysporum* requires functional auxin signalling and transport to promote disease susceptibility (Kidd et al., 2011). Current studies support the dual role of auxin during infection, either by enhancing disease susceptibility (Djami-Tchatchou et al., 2020; Fu and Wang, 2011; Mutka et al., 2013) or increasing resistance (Llorente et al., 2008). There is only little evidence whether bacterial and fungal pathogens directly target key regulators of the auxin signalling pathway. To the best of our knowledge, so far, there was only one study demonstrating that the type III effector AvrRpt2 from *P. syringae* stimulates the degradation of the Aux/IAA protein AXR2 which is a negative regulator in auxin signalling in *A*. *thaliana* (Cui et al., 2013). The degradation of AXR2 promotes pathogenicity, but it remains to be shown whether AXR2 directly interacts with AvrRpt2.

To sum up, it has become evident that successful virus infections result from compatible interplays between plant viruses and phytohormones including auxin signalling. Some viruses such as TMV, RDV and BNYVV inactivate negative regulators of auxin signalling, whereas other

viruses such as SRBSDV, RSBSDV, RSMV and RSV target positive regulators (transcriptional activators) of auxin signalling. Only recently, it was discovered that the P22 protein from *Tomato chlorosis virus* binds to the C-terminal part of SKP1.1 and destabilize the SCF^{TIR1} complex assembly resulting in a suppression of Aux/IAA degradation and promoting virus infection (Liu et al., 2021). This finding adds a new molecular mechanism as the SCF^{TIR1} complex mediating protein degradation via the ubiquitin pathway is targeted by a plant virus to disrupt auxin signalling. As indicated above, transcriptional changes in auxin-responsive genes have been also observed in other plant-virus pathosystems for which a direct interaction between viral proteins and regulators of auxin signalling have not been elucidated yet. Therefore, how viral infections precisely reprogram and regulate auxin-mediated responses is far from being understood which represents one of important future research directions. One main obstacle in finding a putative interaction is on the hand the diversity of viral proteins and on the other hand the large number of plant proteins involved in auxin signalling which results in a high number theoretical interactions. This problem can only be overcome by comprehensive protein–protein interaction screening. Elucidation of exact roles of auxin signalling pathways in host defense response and mechanisms of their subversion by viruses for the pathogen benefit will improve our understanding of plant-virus interactions and assist in development of novel antiviral strategies, *e.g*. identification of the key residues in the host protein interacting domains for genetic intervention (gene editing, plant breeding). It has been shown for some of the aforementioned viruses that a loss of the interaction with the auxin signalling pathway correlates with increased host resistance. Creating recessive resistance using the CRISPR/Cas9 technology to prevent the interaction with key regulators of the auxin signalling pathway could help to develop control strategies.

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Manuscript II

The virulence factor p25 of Beet necrotic yellow vein virus interacts with multiple Aux/IAA proteins from *Beta vulgaris*: implications for rhizomania development

Maximilian M Muellender[1], Eugene I. Savenkov[2], Michael Reichelt[3], Mark Varrelmann[1], Sebastian Liebe[1*]

[1]Institute of Sugar Beet Research, Department of Phytopathology, Göttingen, Germany

[2]Department of Plant Biology, Uppsala BioCenter SLU, Swedish University of Agricultural Sciences, Linnean Center for Plant Biology, Uppsala, Sweden

[3]Max Planck Institute for Chemical Ecology, Department of Biochemistry, Jena, Germany

***Correspondence:** Liebe, Sebastian <liebe@ifz-goettingen.de>

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Abstract

Rhizomania caused by Beet necrotic yellow vein virus (BNYVV) is characterized by excessive lateral root (LR) formation. Auxin-mediated degradation of Aux/IAA transcriptional repressors stimulates gene regulatory networks leading to LR organogenesis and involves several Aux/IAA proteins acting at distinctive stages of LR development. Previously, we showed that BNYVV p25 virulence factor interacts with BvIAA28, a transcriptional repressor acting at early stages of LR initiation. The evidence suggested that p25 inhibits BvIAA28 nuclear localization, thus, de-repressing transcriptional network leading to LR initiation. However, it was not clear whether p25 interacts with other Aux/IAA proteins. Here, by adopting bioinformatics, *in vitro* and *in vivo* protein interaction approaches we show that p25 interacts also with BvIAA2 and BvIAA6. Moreover, we confirmed that the BNYVV infection is, indeed, accompanied by an elevated auxin level in the infected LRs. Nevertheless, expression levels of BvIAA2 and BvIAA6 remained unchanged upon BNYVV infection. Mutational analysis indicated that interaction of p25 with either BvIAA2 or BvIAA6 requires full-length proteins as even single amino acid residue substitutions abolished the interactions. Compared to p25-BvIAA28 interaction that leads to redistribution of BvIAA28 into cytoplasm, both BvIAA2 and BvIAA6 remained confined into the nucleus regardless of the presence of p25 suggesting their stabilization though p25 interaction. Overexpression of p25-interacting partners (BvIAA2, BvIAA6 and BvIAA28) in *Nicotiana benthamiana* induced an auxin-insensitive phenotype characterized by plant dwarfism and dramatically reduced LR development. Thus, our work reveals a distinct class of transcriptional repressors targeted by p25.

Introduction

BNYVV belongs to the genus *Benyvirus* within the family *Benyviridae* and is the causal agent of rhizomania disease in sugar beet (Tamada *et al.*, 1989; Tamada & Abe, 1989; Gilmer *et al.*, 2017). Rhizomania was first described in Italy in the early 1950s and spread to almost all sugar beet-growing areas worldwide in the past decades (McGrann *et al.*, 2009; Liebe *et al.*, 2016). The virus causes leaf symptoms, such as yellowing and vein necrosis. Most important, however, are the severe symptoms induced in the infected taproots characterized by reduced size and wineglass shape, necrosis of the vascular tissue and massive lateral root (LR) proliferation, termed as root beard (Tamada & Abe, 1989). These root symptoms cause

dramatic reduction of taproot weight and massive yield losses, making BNYVV to be one of the most important viral pathogens in sugar beet cultivation. BNYVV is naturally transmitted by the soil-borne plasmodiophoromycete *Polymyxa betae* Keskin which can persist in soil for decades (Tamada & Kondo, 2013). Nowadays, the only efficient way to control rhizomania disease is the cultivation of resistant sugar beet varieties.

BNYVV has a multipartite genome comprising four to five positive-sense, single-stranded RNA segments. Each RNA is capped at the 5' end and polyadenylated at the 3' end. RNA1 possesses one open reading frame (ORF) encoding an RNA-dependent RNA polymerase with motifs for methyltransferase, helicase and a papain-like protease (Bouzoubaa *et al.*, 1987; Richards & Tamada, 1992; McGrann *et al.*, 2009). RNA2 contains six ORFs, encoding a coat protein (CP), a CP-read-through (CP-RT) protein, a triple gene block (TGB) of movement proteins and a small 14 kDa cysteine-rich protein, a viral suppressor of RNA silencing (Tamada & Kusume, 1991; Chiba *et al.*, 2013). RNA3 encodes the p25 protein, the virulence factor that is required for systemic infection in *Beta* species and symptom development (Tamada *et al.*, 1989; Koenig *et al.*, 1991; Lauber *et al.*, 1998).

The massive proliferation of lateral roots (LR) upon BNYVV infection relies on the presence of p25 (Koenig *et al.*, 1991; Tamada *et al.*, 1999; Peltier *et al.*, 2011). In general, the development of LRs is controlled by the phytohormone auxin and its tightly regulated transport and signaling pathways (Gray *et al.*, 2001; Dharmasiri *et al.*, 2005; Ori, 2019). Aux/IAA proteins are key regulators within this auxin signaling pathway as they inhibit the transcriptional activity of auxin response factors (ARFs) under low auxin concentration (Luo *et al.*, 2018). In turn, ARFs are transcription factors regulating the expression of auxin-responsive genes by binding to auxin-responsive elements (AREs) within the promotors (Chandler, 2016; Li *et al.*, 2016). Aux/IAA proteins are rapidly degraded when the cellular auxin level increases. This leads to a release of ARFs, regulating the expression of auxin-responsive genes (Leyser, 2018). Interestingly, the sugar beet taproot undergoes massive reprogramming of auxin-responsive genes upon BNYVV infection (Schmidlin *et al.*, 2008; Gil *et al.*, 2018; Gil *et al.*, 2020). This includes the LATERAL ORGAN BOUNDARIES DOMAIN (LBD) transcriptional network as well as expression of EXPANSINS (EXPs), all of which are important for LR development (Liu *et al.*, 2005a; Lee & Kim, 2013; Lee *et al.*, 2015). In a sugar beet cDNA library screen (Thiel & Varrelmann, 2009), we identified the Aux/IAA protein BvIAA28 (also known as BvAUX28) as a

putative interaction partner of p25. Further characterization showed that p25 interacts with BvIAA28 via domains I and II (Gil *et al.*, 2018). Additionally, the co-expression of both proteins revealed that p25 inhibits the nuclear localization of BvIAA28. It has been assumed that the p25-mediated translocation of BvIAA28 into the cytoplasm deprives the protein of its repressor activity in the nucleus leading to an up-regulation of auxin-responsive-genes that are under the control of BvIAA28.

The discovery that the p25 virulence factor interacts with a sugar beet Aux/IAA protein (BvIAA28) (Thiel & Varrelmann, 2009; Gil *et al.*, 2018) prompted us to test the other BvAux/IAAs proteins for their potential interaction with p25 employing three independent methods, namely, yeast two-hybrid system (Y2H), bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (co-IP). This study identified two additional Aux/IAA proteins − BvIAA2 and BvIAA6 − interacting with p25. Further analysis revealed that p25 sequesters negative regulators of LR initiation and development suggesting activation of a transcriptional network leading to LR induction. This study expands the repertoire of the p25-interacting partners and their potential role in development of rhizomania syndrome.

Material and Methods

BNYVV sugar beet inoculation

The BNYVV susceptible sugar beet genotype KWS03 (KWS Saat SE, Einbeck, Germany) was used for infection with BNYVV. Young sugar beet seedlings were mechanically inoculated with the BNYVV A-type infectious clone (Laufer *et al.*, 2018b) according to (Liebe *et al.*, 2020). All plants were kept under controlled greenhouse conditions (24 °C/14 hr light, 18 °C/10 hr dark). BNYVV infection and measurement of relative virus contraction in lateral roots was determined by means of DAS-ELISA (DSMZ, AS-0737, Brunswick, Germany) as described by (Liebe *et al.*, 2020).

Auxin quantification

To measure the auxin content in healthy and BNYVV infected sugar beet roots, 250 mg of homogenized root cortex and lateral root tissue per sample was used. Auxin (indole-3-acetic acid) was extracted with 1 ml methanol containing 40 ng of D5-indole-3-acetic-acid (OlChemIm s.r.o, Olomouc, Czech Republic) at 42 and 66 dpi. The experiment was performed

in eight biological replicates. Samples were analyzed using liquid chromatography (Agilent 1260 Infinity Quaternary LC system, Agilent Technologies, Santa Clarita, California) coupled to a triple quadrupole mass spectrometer (LC-MS/MS). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6mm, 1.8µm, Agilent Technologies). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B respectively. The elution profile was: 0-0.5min, 10% B in A; 0.5-4min, 10-90% B in A; 4.1-4.5min 100% B and 4.6-7min 10% B in A. The mobile phase flow rate was 1.1 ml min^{-1}. The column temperature was maintained at 25 °C. The liquid chromatography was coupled to a QTRAP 6500 tandem mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with a Turbospray ion source operated in positive ionization mode. The ionspray voltage was maintained at 5500 eV. The turbo gas temperature was set at 650 °C. Nebulizing gas was set at 60psi, curtain gas at 40psi, heating gas at 60psi and collision gas at medium. Multiple reaction monitoring was used to monitor analyte parent ion → product ion: m/z 176 → 130 for indol-3-acetic acid; m/z 181 →133 + m/z 181 →134 + m/z 181 →135 for D5-indol-3-acetic acid. Collision energy was 19V; declustering potential was 20V. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing.

Yeast two-Hybrid

To identify protein-protein interaction of p25 with the Aux/IAA Proteins from *B. vulgaris*, a yeast two-hybrid system (YTH) was used (Fields & Song, 1989). After RNA extraction from BNYVV susceptible sugar beet root material (MACHEREY-NAGEL, Dueren, Germany) and subsequent cDNA synthesis (Thermo Fisher Scientific, Waltham, Massachusetts) all sugar beet encoded *Aux/IAA* genes were based on the annotated sequence from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Table S1). The *Aux/IAA* genes were cloned into pJG4-5 vectors with C-terminal B42 transcription activation domain-HA epitope (AD-Aux/IAA) as prey. The viral pathogenicity factor from BNYVV, *p25* was cloned into pEG202 with CDS1 LexA DNA binding domain (BD-p25) as bait. All plasmids were generated using standard restriction enzyme cloning (Thermo Fisher Scientific). After transformation into chemically competent DH5*α E. coli* cells (Inoue *et al.*, 1990), all plasmids were verified by commercial capillary Sanger sequencing (Microsynth Seqlab, Goettingen, Germany). The constructs were super transformed into the high sensitivity strain *S. cerevisiae* EGY48: MATα, *trp1*, *his3*, *ura3*, *leu2*::*6*

LexAop-LEU2 using a lithium acetate/single-stranded carrier DNA/polyethylene glycol method (Gietz & Woods, 2002). The GFP plasmid pGNG1 was omitted, because no screen was performed to identify unknown interaction partners and it was not necessary to select for green florescent colonies. All recipes were taken from the Origene *DupLEX-A* user's manual and modified according to the individual requirements. The lacking amino acids in the drop out media were indicated by the single-letter amino acid code. BD-p25 with each AD-Aux/IAA AD-Aux/IAA were co-transformed to test for interaction and BD-p25 or BD-IAA transformed with the AD or BD without any fusion proteins, respectively, served as control for autoactivation. Three colonies were then individually resuspended according to protocol and diluted in water. Then, 5 µl of the dilution series (1×10^{-1}-1×10^{-4}) was spotted on DOBA (gal/raf) -H, -W as growth control, DOBA (gal/raf) -H, -W, -L as interaction or as autoactivation medium, respectively. The positive control AD-p53 with BD-LTA and the negative control AD(-empty) with BD(-empty) were supplied by MoBiTech (Göttingen, Germany). The growth controls were incubated at 30 °C for about 3-4 days, the interaction- and autoactivation controls for about 5-6 days.

Preparation of *R. radiobacter* for agroinoculation

Electrocompetent cells of the *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*/ *Agrobacterium fabrum*) strain C58/C1 were used for transformation of all plasmids, used in this work (Voinnet *et al.*, 1998).

Bimolecular fluorescence complementation assay

To verify the results from YTH, bimolecular fluorescence complementation assay (BiFC) was used according to (Jach *et al.*, 2006; Zilian & Maiss, 2011). The Aux/IAA candidates were fused C- and N-terminally to the N-terminal part of mRFP (mRFPN) and p25 was fused in both orientations to mRFPC by one-step cloning isothermal Gibson assembly (Gibson *et al.*, 2009). The constructs were inoculated with an OD_{600} of 0.7 into leaves of four- to five-week-old *N. benthamiana* wild type plants. Fluorescence in the leaf patches was assessed microscopically at 4 dpi by epifluorescence microscopy at 4 dpi. Positive and negative controls were taken from the BiFC assay (Zilian & Maiss, 2011).

Co-immunoprecipitation

For final confirmation of the protein interaction results, *in planta* co-immunoprecipitation (co-IP) was chosen. Both Aux/IAA proteins IAA2 and IAA6 and p25 were cloned into the plant expression vector pDIVA (Acc. No. KX665539) under control of CaMV 35S promotor. Additionally, mutants encoding degradation resistant protein variants (BvIAA2 $_{P162L}$, BvIAA6 $_{P64L}$) allowing higher protein accumulation were created by PCR mutagenesis and subsequent sequencing (Worley *et al.*, 2000). These protein variants were generating using PCR mutagenesis and confirmed by sequencing. To further increase the expression of the Aux/IAAs, a Tobacco etch virus (TEV) translational enhancer sequence (Zilian & Maiss 2011) was inserted upstream of the *Aux/IAA* genes. The Aux/IAA proteins were fused to the N-terminus of a 3xFLAG tag (DYKDDDDK) and a single HA tag (YPYDVPDYA) was fused to the p25 C-terminus. Three days after infiltration of *N. benthamiana* leaves with the constructs, the patches were harvested and grounded in liquid nitrogen to a fine powder. The powder was mixed 1:1 (w/v) with extraction buffer (Sacco *et al.*, 2007; Sohn *et al.*, 2014) supplied with 50 µM Mg132 (Sigma-Aldrich, St. Louis, Missouri) to prevent proteasome mediated protein degradation. After incubation on ice for 5 min, the reaction tubes were centrifuged (5000 g) for 15 min at 4 °C and the supernatant was used as input. For immunoprecipitation, 500 µl of the input was mixed with 25 µl equilibrated Pierce™ Anti-HA Magnetic Beads (Thermo Fisher Scientific, Waltham, Massachusetts) and incubated for 1 hour at 4 °C. After three washing steps, all bound proteins to the beads were eluted using 2x Laemmli buffer (Bio-Rad Laboratories, Hercules, California) and incubated for 5 min at 95 °C. Additionally, the proteins were detected in the input. All samples were checked using SDS polyacrylamide gel electrophoresis and immunoblotting.

Subcellular localization

To determine the subcellular localization of BvIAA2 and BvIAA6, both genes were fused by Gibson assembly to GFP containing an HA tag. To investigate the effect of p25 on the subcellular localization of Aux/IAAs, only an HA tag was added to minimize negative effects of long attachments. Additionally SV40 NLS was fused to dsRed and served as plant nuclear marker (Kalderon *et al.*, 1984; Lassner *et al.*, 1991). The plasmid pDIVA was used as backbone for the cloning of the localization plasmids (Laufer *et al.*, 2018b). Co-expression was performed

by means of agroinfiltration with an OD_{600} of 0.7 into *N. benthamiana* leaves. Fluorescence in the leaf patches was assessed microscopically at 4 dpi. The HA tag used to verify protein expression via immunodetection.

Domain mapping of p25 and interacting Aux/IAA proteins

To identify interacting domains of p25 A-type with Aux/IAA proteins, five amino acids were randomly inserted over the whole protein using the Mutation Generation System Kit (F701 - Thermo Fisher Scientific). To check for interacting domains of the Aux/IAA proteins, the previously described domains were used (DI-DIV). BvIAA2 and BvIAA6 were separated into two parts (DI-II and DIII-IV) and the described domains were deleted individually (DII-IV; DI, III, IV; DI, II, IV; DI-III). For IAA2 primers were designed to delete the domains between amino acid positions AA 102/103, AA 189/190 and AA 252/253 and the primers for IAA6 were designed to delete the domains between amino acids AA 41/42, AA 76/77 and AA 132/133. The deletions were introduced into the respective YTH and BiFC plasmids by PCR mutagenesis with subsequent sequencing.

Confocal laser scanning microscopy

To visualize protein fluorescence, confocal laser scanning microscopy (CLSM) was used. The mRFP and GFP fluorescence was visualized with the TCS-SP5 confocal laser-scanning microscope (Leica Microsystems). Excitation/emission wavelengths for mRFP were 566 nm/515–523 nm and for GFP the wavelengths were 488 nm/515–523 nm. All confocal images were processed with the LAS-AF software version 2.6.3.8173 (Leica Microsystems, Wetzlar, Germany).

Protein extraction from yeast and plant tissue, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and immunodetection

Protein extraction from yeast was carried out as described by and protein extraction of total plant proteins was carried after (Thiel & Varrelmann, 2009). All protein samples were separated by 12% SDS polyacrylamide gel electrophoresis and electroblotted on polyvinylidene diflouride membranes (Roche, Basel, Switzerland) using semi-dry blotting system (Bio-Rad Laboratories). Immunodetection of HA was carried out using anti-HA high-affinity rat monoclonal antibody (Merck KGaA, Burlington, Vermont - 11 867 423 001, 1:1,000)

and alkaline phosphatase (AP) conjugated goat anti-rat immunoglobulin G (IgG) (whole molecule) (Merck KGaA - A8438, 1:10,000). FLAG (Merck KGaA - F7425, 1:1,000) and LexA (Merck KGaA - 06-719, 1:2,500) were detected with polyclonal rabbit antibodies and AP-conjugated goat anti-rabbit polyclonal antibodies (Merck KGaA - A3687, 1/10,000). C-myc (EQKLISEEDL) was probed with anti-C-myc mouse monoclonal IgG (Thermo Fisher Scientific - 13-2,500, 1:500) and detected with AP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania - 115-055-003, 1:10,000). Signal detection was performed using NBT/BCIP (chromogenic substrates nitroblue tetrazolium chloride and 5-bromo-4-chloro-3'-indolyphosphate) ready-to-use tablets (Merck KGaA).

Heterologous expression of Aux/IAA proteins

For heterologous expression of Aux/IAA proteins, the genes from BvIAA2, BvIAA6 and BvIAA28 were cloned into the infectious TRV RNA2 cDNA clone by Gibson assembly under the control of a subgenomic promoter of the Pea early-browning virus downstream of the TRV-CP (Liu *et al.*, 2002; Ghazala & Varrelmann, 2007; Lindbo, 2007). Additionally, degradation resistant protein variants (BvIAA2 $_{P162L}$, BvIAA6 $_{P64L}$, BvIAA28 $_{P146L}$), allowing higher protein accumulation, were generated by PCR mutagenesis (Worley *et al.*, 2000). A TRV RNA2 expressing mRFP (RNA2-mRFP) was used as control to distinguish symptoms of candidate gene overexpression from general TRV symptoms and to check for systemic infection. For systemic TRV infection, the leaves of 14-days-old *N. benthamiana* seedlings were inoculated with an OD_{600} of 0.5 of RNA1 and each RNA2 construct. The root and leaf phenotypes were examined at 33 days post infection (dpi). A systemic TRV infection was confirmed by RNA extraction, cDNA synthesis, and final PCR of heterologous expressed Aux/IAA proteins in *N. benthamiana* leaves.

RT-qPCR analysis

BvIAA2, *BvIAA28* and *BvIAA6* expression was relatively quantified in cDNA of the LR tissue of sugar beet using reverse transcriptase quantitative PCR (RT-qPCR). After RNA extraction (MACHEREY-NAGEL) and cDNA synthesis (Thermo Fisher Scientific) samples were analyzed using iTaq™ Universal SYBR® Green Supermix (#1725121 - Bio-Rad Laboratories). Oligonucleotides were designed with NCBI primer-BLAST (listed in Table S2). The expression of both *Aux/IAA* genes was quantified relative to the housekeeping genes *glyceraldehyde 3-*

phosphate dehydrogenase (*GAPDH*, XM_010679634.2) and *elongation factor 1 β* (*EEF1B2*, NM_001303081.2). All qPCR reactions were performed with a C1000 Touch™ Thermal cycler equipped with a CFX96™ Real Time System (Bio-Rad Laboratories). RT-qPCR conditions were as follows: an initial denaturation of 95 °C for 3 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 20 s, 72 °C for 30 s, final extension of 72 °C for 5 min. All three biological replicates were analyzed in two technical replicates. The Ct values and curves for analysis were generated by the CFX Manager™ Software (Bio-Rad Laboratories) and data normalization and calculation of relative expression values was done using the $2^{-\Delta Ct}$ method (Livak & Schmittgen, 2001). The statistical independence between root tissue and leaf tissue was calculated for the individual ΔCt values at each time point.

Bioinformatic analysis

Multiple protein sequences alignments, as well as maximum likelihood trees were generated using Geneious 2020.1 software (default settings - Biomatters). Protein sequences of the *B. vulgaris* (BvIAA) and *A. thaliana* (AtIAA) Aux/IAA proteins were downloaded from KEGG database and alignments of sequences were generated using the ClustalW algorithm (default settings - Biomatters).

Statistical analysis

Statistical analysis was performed with SigmaPlot14 (SigmaPlot 14.0, Systat Software Inc.). The data were first tested for normal distribution ($p \leq 0.05$) using Kolmogorov-Smirnov test followed by Brown-Forsythe test to check for equality of group variances ($p > 0.05$). The data were analyzed using Student's t-test. When equality of variances cannot be assumed, Welch's t-test was used. Graphic representations of the data were created using Excel 2013 (Microsoft Corp.). In each graph, the standard deviation (SD) and significance (not significant (n.s.) = $p > 0.05$; * = $0.01 \leq p < 0.05$; ** = $0.001 \leq p < 0.01$; *** = $p < 0.001$) are displayed. Significant differences between several variants on one factor were performed using one-way ANOVA. Data in tables are presented as mean values ±SD (standard deviation).

Results

Phylogenetic and functional analysis of the Aux/IAA proteins from *B. vulgaris*

The Aux/IAA protein BvIAA28 interaction with p25 was previously identified from a screening using a sugar beet cDNA library prepared from a resistant genotype that prevents efficient virus replication and massive lateral root proliferation upon BNYVV infection (Thiel & Varrelmann, 2009). Furthermore, the massive transcriptional reprogramming of auxin-responsive genes observed in a susceptible genotype (Gil *et al.*, 2020) prompted us to hypothesize that p25 might interact with numerous Aux/IAAs. Therefore, to address this hypothesis, we set up a screen with all known Aux/IAAs from sugar beet. Using the KEGG GENOME database, 12 potential Aux/IAA candidates were identified (Table S1). Transcripts of the candidate genes from a BNYVV susceptible genotype were sequenced and a multiple sequence alignment and phylogenetic analysis performed. The alignment clearly showed the presence of all four canonical Aux/IAA domains in all candidates except for two. BvIAA4.2 and BvIAA33 appeared not to contain domain II and, thus, were assigned to the class of non-canonical Aux/IAA proteins (Figure 1A). A maximum likelihood tree of the Aux/IAA candidates from *B. vulgaris* together with all known 29 Aux/IAA proteins from *Arabidopsis thaliana* was computed to define potential clades and orthologous groups based on the similarity to corresponding Arabidopsis proteins (Liscum & Reed, 2002; Overvoorde *et al.*, 2005; Luo *et al.*, 2018). Most BvIAA proteins clustered together with the corresponding *Arabidopsis* proteins into ten clades of putative functional homologs (Figure 1B). Notably, BvIAA2, BvIAA4, BvIAA6, BvIAA8, BvIAA9, BvIAA13, BvIAA14 and BvIAA28 proteins clustered together with *Arabidopsis* Aux/IAA proteins involved in root development (Figure 1B, green circles), only BvIAA29 and BvIAA33 fell into other clades (Reed, 2001; Luo *et al.*, 2018). Nevertheless, we tested all Aux/IAA proteins from sugar beet for interaction with p25.

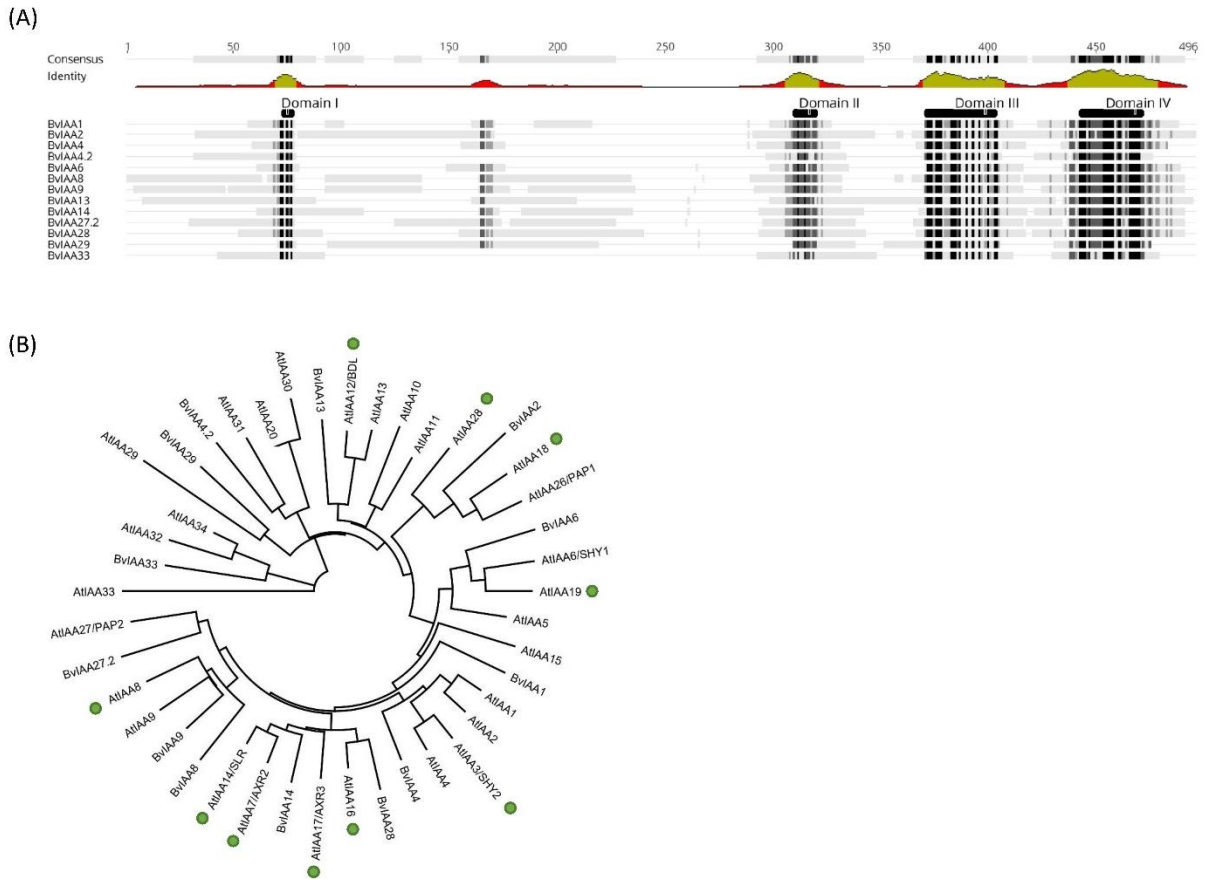

Figure 1. (A) Multiple sequence alignment (Geneious 2020.1 software) of all IAA proteins from sugar beet (BvIAA). Black and grey shades in the sequences and green regions of graph below the consensus indicate a high homology of the protein sequences. The functional domains of the proteins are also shown above the sequences (DI-DIV). **(B)** Maximum likelihood tree of all BvIAA proteins and all Aux/IAA proteins from *A. thaliana* (AtIAA). Proteins involved in root formation in *A. thaliana* are highlighted with green circles (after Reed, 2001; Luo et al., 2018).

Interaction studies of p25 with the Aux/IAA proteins from *B. vulgaris*

To determine whether the Aux/IAA from sugar beet interact with p25, a Y2H experiment was performed. The analysis revealed that BvIAA2, BvIAA6, BvIAA13, BvIAA14, BvIAA29 and BvIAA33 could potentially interact with p25 and none of these proteins displayed autoactivation of Y2H-inducible reporter (Figure 2). The six interactors were selected for further validation using bimolecular fluorescence complementation (BiFC) (Zilian & Maiss, 2011) in *N. benthamiana* leaf tissue. The BiFC experiments showed that among the six candidates tested, only BvIAA2 and BvIAA6 interact with p25 *in planta*. Moreover, these interactions could only be detected when p25 was fused C-terminally to mRFP-C and the Aux/IAA candidates were fused N-terminally to mRFP-N (Figure 3A). Co-expression of the abovementioned BiFC constructs with the nuclear marker GFP-SV40 revealed that the

interactions of p25 with both Aux/IAAs are strongly restricted to the nucleus (Figure 3C). The interactions of p25 with BvIAA2 and BvIAA6 were also confirmed by co-IP experiments in *N. benthamiana* leaves (Figure 3D). However, only the interaction of p25 with the degradation-resistant variants of the Aux/IAA proteins could be detected (Figure 3D). Notably, in the input samples, the accumulation of the unmodified wt Aux/IAA proteins was much low compared to the degradation-resistant variants suggesting fast turnover of the BvIAA2 and BvIAA6 proteins as expected. Finally, the expression of all BvIAA proteins tested and p25 in all three assays was confirmed by immunoblotting (Figure S1, S2).

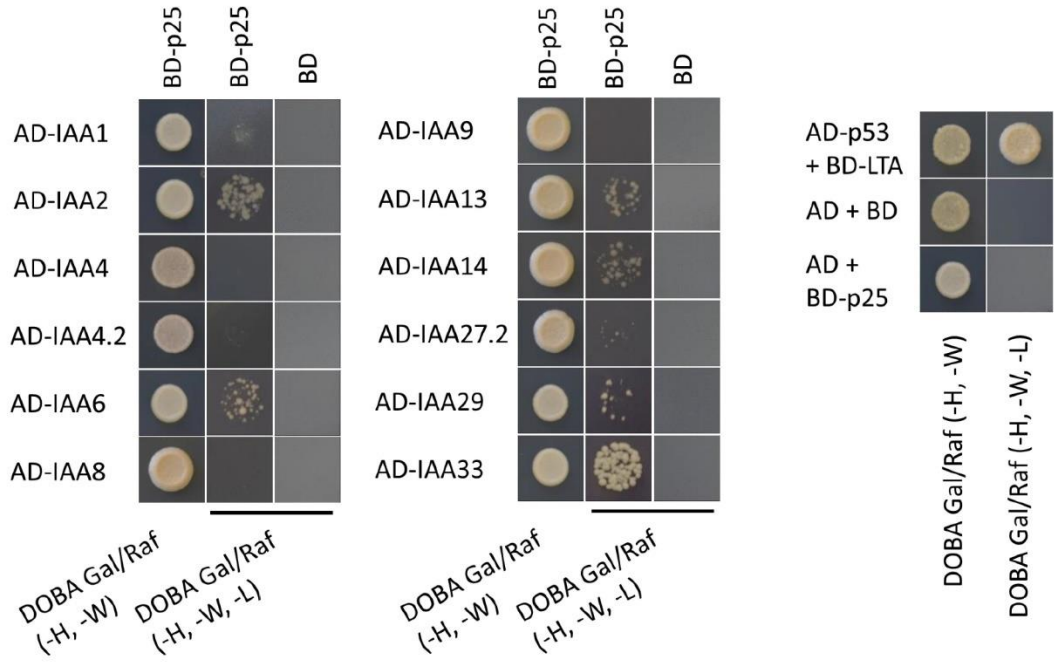

Figure 2. Results from YTH experiment with all Aux/IAA proteins from sugar beet and BNYVV p25. The positive control AD-p53 with BD-LTA and the negative control AD(-empty) with BD(-empty) were supplied by MoBiTech. BNYVV p25 was fused to the BD and the IAAs to the AD to test for interaction. Yeast transformants, containing both plasmids were selected on DOBA Glu (-H, -W), single colonies were resuspended in water and diluted 1×10^{-1}- 1×10^{-4}. 5 µl of each dilution was spotted on the control medium (DOBA Glu (-H, -W) and selection medium (DOBA Gal/Raf (-H, -W, -L)), only the 1×10^{-2} dilution is shown here. An AD or BD without any fusion proteins and transformed with BD-p25 or AD-Aux/IAA, respectively, served as control for autoactivation. AD - activating domain; BD - binding domain; DOBA – Dropout Base Agar.

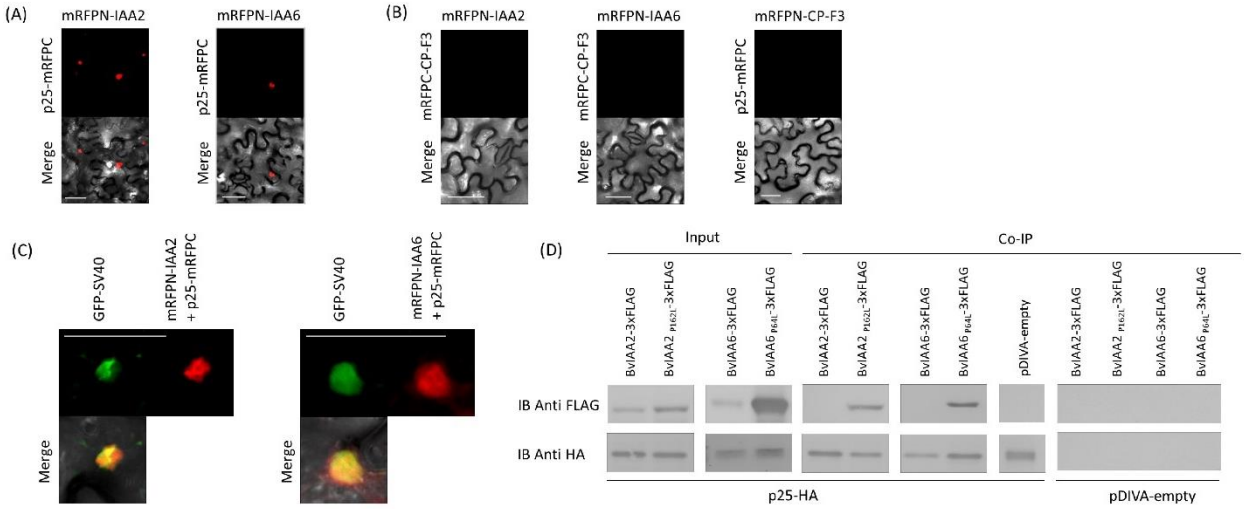

Figure 3. Confirmation of the BNYVV p25 interaction with IAA2 and IAA6 by bimolecular fluorescence complementation and co-immunoprecipitation. The candidates were co-expressed in *N. benthamiana* leaves by *A. tumefaciens* C58C1 cells harboring **(A)** pCB:p25-mRFPC/pBiFC-mRFPN-IAA2 or pCB:p25-mRFPC/pCB:mRFPN-IAA6 to test the interaction and **(B)** pCB:mRFPC-CP-F3/pCB:mRFPN-IAA2, pCB:mRFPC-CP-F3/pCB:mRFPN-IAA2 or pCB:p25-mRFPC/pCB:mRFPN-CP-F3 to test for autoactivation of the fusion proteins. **(C)** Additionally, the interacting BiFC partners were co-expressed with the nuclear marker pDIVA:GFP-SV40 to confirm the nuclear localization of the interaction. Images were taken at 4 dpi. Scale bars, 50 µm. **(D)** Immunoblot (IB) showing the Aux/IAA proteins coimmunoprecipitated with p25. The total proteins were isolated from *Agrobacterium*-infiltrated *N. benthamiana* leaves expressing the Aux/IAAs-3xFLAG (up) and p25-HA (low). The input is shown at the left and the and immunoprecipitated samples with anti-HA antibodies are shown in the right. The candidates, co-infiltrated with pDIVA-empty were used as controls, to detect unspecific binding.

Mapping of interacting sites in p25 and Aux/IAAs

To identify amino acid residues in BvIAA6, BvIAA2 and p25 involved in the protein interactions fourteen *p25* mutants were generated by pentapeptide scanning mutagenesis. The expression of these mutants results in a single five amino acid insertion randomly distributed along the sequence of the protein (Table 1). Seven randomly chosen p25 mutants were tested for interaction with BvIAA2 and nine randomly chosen mutants were assessed for interaction with IAA6 in Y2H system. Two of the mutants were tested with both BvAux/IAA proteins to confirm the validity of the results for both candidates. Surprisingly, these experiments showed that none of the p25 mutants interacted (Table 1) neither with BvIAA2 nor with BvIAA6. To further verify the interaction of p25 with BvIAA2 and BvIAA6, three p25 mutants of those tested above were used in BiFC experiments. The results confirmed the Y2H experiments, no interaction of the p25 mutants with BvIAA2 or BvIAA6 could be detected (data not shown). As before, the protein expression of wild type p25 and the expression of four randomly selected p25 mutants was confirmed by immunoblotting (Figure S3).

Table 1. Results of the YTH assay of the p25 pentapeptide scanning mutants tested for interaction with IAA2 and IAA6.

p25 variety	IAA2	IAA6
p25 wt	✓	✓
p25.Val51_Tyr52ins5	X	n.d.
p25.Gly119_Leu120ins5	X	n.d.
p25.Val130_Pro131ins5	X	n.d.
p25.Val140_Asp141ins5	X	n.d.
p25.Val178_Asn179ins5	X	n.d.
p25.Val81_Met82ins5	X	X
p25.Asp200_Val201ins5	X	X
p25.Cys31_Arg32ins5	n.d.	X
p25.Arg62_Gly63ins5	n.d.	X
p25.Pro93_Ile94ins5	n.d.	X
p25.Asn118_Gly119ins5	n.d.	X
p25.Val121_Ile122ins5	n.d.	X
p25.Leu132_His133ins5	n.d.	X
p25.Asn156_Ala157ins5	n.d.	X

Notes: Checkmark (✓) = positive interaction, cross (X) = no interaction, n.d. = not determined.

To reveal which domains of BvIAA2 and BvIAA6 are required for the interaction with p25, six constructs for each BvIAA protein expressing various sets of the conserved domains I to IV were tested by Y2H assays and BiFC (Table 2). In both experiments, no interaction was detected with either the Y2H test or BiFC (Table 2), showing that deletion of any domain of BvIAA2 or BvIAA6 results in loss of interaction with p25. Similar to the p25 mutants as described above, it was found that deletion of any domain of BvIAA2 or BvIAA6 resulted in loss of interaction with p25. Only wt IAA2 and IAA6 showed stable interaction with p25 in YTH and BiFC (Table 2).

Table 2. Results of the YTH and BiFC assays with the different domain variants of IAA2 and IAA6 with p25 wt.

IAA	YTH	BiFC
IAA2	✓	✓
IAA2 DI+II	X	X
IAA2 DIII+IV	X	X
IAA2 DII, III, IV	X	X
IAA2 DI, III, IV	X	X
IAA2 DI, II, IV	X	X
IAA2 DI, II, III	X	X
IAA6	✓	✓
IAA6 DI+II	X	X
IAA6 DIII+IV	X	X
IAA6 DII, III, IV	X	X
IAA6 DI, III, IV	X	X
IAA6 DI, II, IV	X	X
IAA6 DI, II, III	X	X

Notes: Checkmark (✓) = positive interaction, cross (X) = no interaction.

We then investigated whether alterations (amino acid residue substitutions) in the nuclear localization signal (NLS) and nuclear export signal (NES) (Vetter *et al.*, 2004) of p25 affect the interaction with BvIAA2 and BvIAA6. To this end, the p25 NLS motif ^{57}KRIRFR^{62} was replaced with either ^{57}AAIAFA^{62} or ^{57}KRIRFA^{62} and the NES motif ^{169}VYMVCLVNTV^{178} was altered to ^{169}AYMACLVNTV^{178} (Vetter *et al.*, 2004). The Y2H and BiFC experiments showed that interactions with both BvIAA2 and BvIAA6 were lost when either the NLS or NES signal was disrupted (Figure S4).

Subcellular localization of IAA2 and IAA6 upon co-expression with p25

Since a previous study reported that the interaction of p25 with BvIAA28 results in p25-mediated translocation of BvIAA28 from the nucleus into the cytoplasm (Gil *et al.*, 2018), we investigated whether p25 affects the nuclear accumulation of BvIAA2 and BvIAA6. To minimize protein modifications which can disturb the interaction, the BvIAA2 and BvIAA6

proteins were fused to GFP-HA tag and p25 was fused to a single HA-tag only. Anti-HA antibodies were used to detect the proteins by immunoblotting (Figure S5) and the GFP reporter was employed to determine the subcellular localization of the BvIAA2 and BvIAA6 proteins (Figure 4). Additionally, both BvIAA2 and BvIAA6 proteins were co-expressed with dsRed-SV40 to verify their nuclear localization. The localization experiments showed that both, BvIAA2 and BvIAA6 localize to the nucleus regardless of whether they are transiently expressed on their own or co-expressed with p25. To examine an effect of the interaction on p25, the p25 protein was tagged with GFP and co-expressed with either BvIAA2-mRFP or BvIAA6-mRFP. There was no change in the subcellular localization of p25 in the presence of the Aux/IAA proteins, p25 still localized to the nucleus and the cytoplasm (Figure S6).

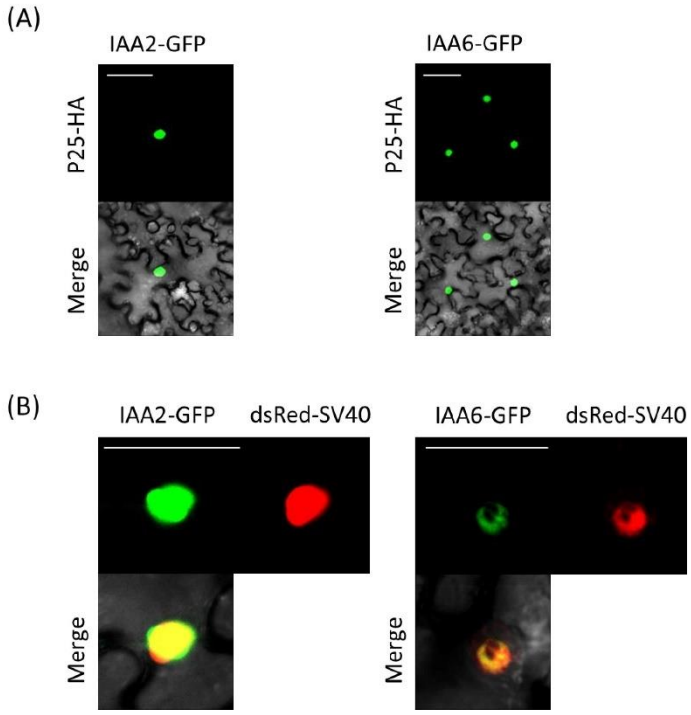

Figure 4. Subcellular localization of interacting Aux/IAAs co-expressed with and without p25 **(A)** Co-infiltration of interacting partners p25 fused to an HA tag (p25-HA) together with IAA2 fused to GFP (IAA2-GFP) or IAA6 fused to GFP (IAA6-GFP). **(B)** Subcellular localization of IAA2-GFP and IAA6-GFP transiently expressed in *N. benthamiana* epidermal leaf cells. Both proteins were co-expressed with the nuclear marker dsRed-SV40. Images were taken at 4 dpi. Scale bars, 50 µm.

Measurement of the indole-3-acetic acid content in BNYVV infected sugar beet plants

Since BNYVV is thought to interfere with important regulatory nodes of the auxin signaling pathway, changes of the auxin concentrations during BNYVV infection process are expected. To address this question, the auxin (indole-3-acetic acid, IAA) content was measured in the root cortex and lateral roots of healthy and BNYVV-inoculated sugar beet plants 42 and 66 dpi using LC-MS/MS. Accumulation of BNYVV in the inoculated plants was confirmed by ELISA (data not shown) prior to measurements and eight biological replicates (individual plants) of each treatment were selected for auxin quantification. The LC-MS/MS measurements revealed that the auxin content in BNYVV infected roots (1.96±0.76 µg g FW^{-1}) was approximately as twice as high compared to healthy sugar beet roots (0.95±0.31 µg g FW^{-1}) at 42 dpi (Student's two-tailed t-test, p = 0.007). In contrast, the auxin content measured in healthy (0.75±0.23 µg g FW^{-1}) and infected (0.69±0.17 µg g FW^{-1}) roots at 66 dpi was similar (Student's two-tailed t-test, $p > 0.05$) (Figure 5).

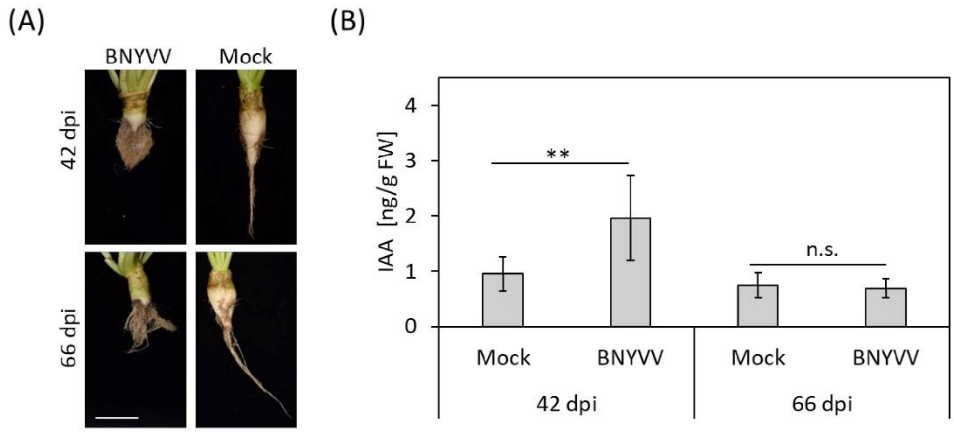

Figure 5. Determination of the IAA content in BNYVV infected sugar beet roots at 42 and 66 dpi by LC-MS/MS. **(A)** Root phenotype of BNYVV mechanically infected vs. non-inoculated (mock) sugar beets after both harvest dates. Scale bar, 5 cm. **(B)** IAA content in the lateral roots and root cortex of BNYVV infected and non-inoculated sugar beet plants. Horizontal bars indicate significance (n.s. = not significant) and vertical bars indicate standard deviation, (n=8).

Quantification of *IAA2* and *IAA6* expression in BNYVV infected sugar beet plants

Having determined that auxin levels are significantly increased in the BNYVV-infected LR, we next asked whether this dramatic change results in altered expression of *BvIAA2* and *BvIAA6.* To address this question, RT-qPCR was conducted for *BvIAA2*, *BvIAA6* and *BvIAA28* using total RNAs of mock-inoculated and virus-infected sugar beet roots at 28, 42 and 66 dpi. But before setting up RT-qPCR experiments, the accumulation of BNYVV in LR of the sugar beet plants selected for RT-qPCR analysis was confirmed by ELISA (Figure S7). There was no change detected in the expression of either *BvIAA2* or *BvIAA6* at any time point tested (Student's two-tailed t-test, $p > 0.05$; Figure 6). Thus, we concluded that the expression of *BvIAA2*, *BvIAA6* and *BvIAA28* was not affected by BNYVV infection.

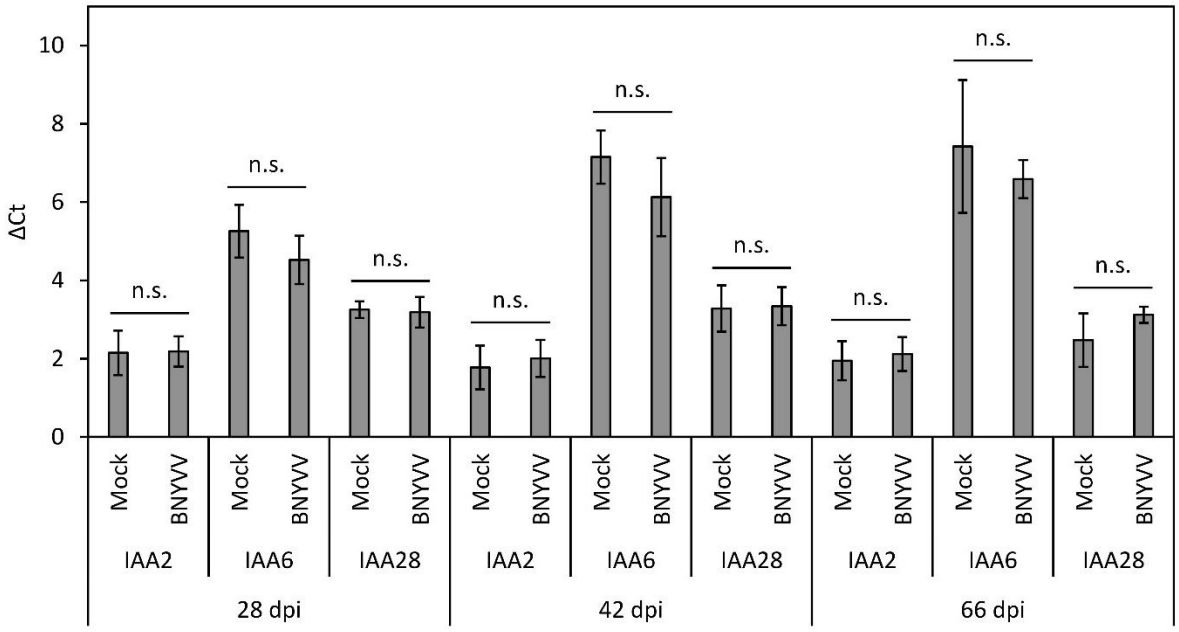

Figure 6. Expression level of *BvIAA2*, *BvIAA6* and *BvIAA28* in BNYVV mechanically infected sugar beet roots compared to the expression in non-inoculated (mock) sugar beet roots. The roots were analyzed at 28, 42 and 66 dpi. Horizontal bars indicate significance (n.s. = not significant) and vertical bars indicate standard deviation, (n=4).

Effect of *BvIAA2*, *BvIAA6* and *BvIAA28* expression on LR formation in *N. benthamiana*

To elucidate a possible effect of the p25-interacting Aux/IAA proteins (BvIAA2, BvIAA6 and BvIAA28) on LR development we overexpressed *BvIAA2*, *BvIAA6* and *BvIAA28* and characterized the *Aux/IAA*-overexpression phenotypes. Initially, we also planned to perform Aux/IAA-knock down experiments in sugar beet using Tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) system. Unfortunately, TRV RNA2 failed to accumulate in sugar

beet inoculated roots and only TRV RNA1 was detectable (data not shown) making VIGS or overexpression from a viral vector not possible in sugar beet. Since *N. benthamiana* represents a more genetically tractable model than sugar beet, and both react to changes in auxin-signaling, all subsequent experiments were performed in *N. benthamiana*. Thus, *BvIAA2*, *BvIAA6* and *BvIAA28* were expressed from TRV vector in *N. benthamiana*. Additionally, the degron motif in domain II of BvIAA2, BvIAA6 and BvIAA28 was altered by site-directed mutagenesis to reduce the auxin mediated degradation of the corresponding proteins and to enhance the phenotypic effect of the overexpression (Worley *et al.*, 2000). The obtained *Aux/IAA* mutants were expressed from TRV vector as well. As expected, heterologous expression of BvIAA2, BvIAA6 and BvIAA28 in *N. benthamiana* resulted in phenotypes that resemble auxin-insensitivity characterized by overall dwarfism of the plant (Park *et al.*, 2002) (Figure 7A). The plant height, number of flowers and root mass was significantly reduced (Student's two-tailed t-test, $p < 0.05$; Figure 7A) as compared to the TRV-mRFP-infected controls. The plants did not differ significantly (Student's two-tailed t-test, $p > 0.05$; Figure 7A) in any of the traits examined (Figure 7B-E), when phenotypes were compared between various constructs (TRV-*BvIAA2* versus TRV-*BvIAA6* versus TRV-*BvIAA28*). Systemic infections of the plants with the corresponding TRV constructs and stability of the insertions were confirmed by PCR (Figure S8B) and sequencing (data not shown).

Expression of the degradation-resistant variants of the Aux/IAA proteins, namely, BvIAA2 $_{P162L}$ and BvIAA8 $_{P146L}$ resulted in death of the plants (Figure S8A). However, plants infected with TRV-*BvIAA6* $_{P64L}$ survived and were characterized by more severe phenotype compared to those induced by TRV-*BvIAA6* (Figure S8A). Hence, the auxin-insensitivity phenotype already observed with the unmodified BvIAA6 could be further enhanced.

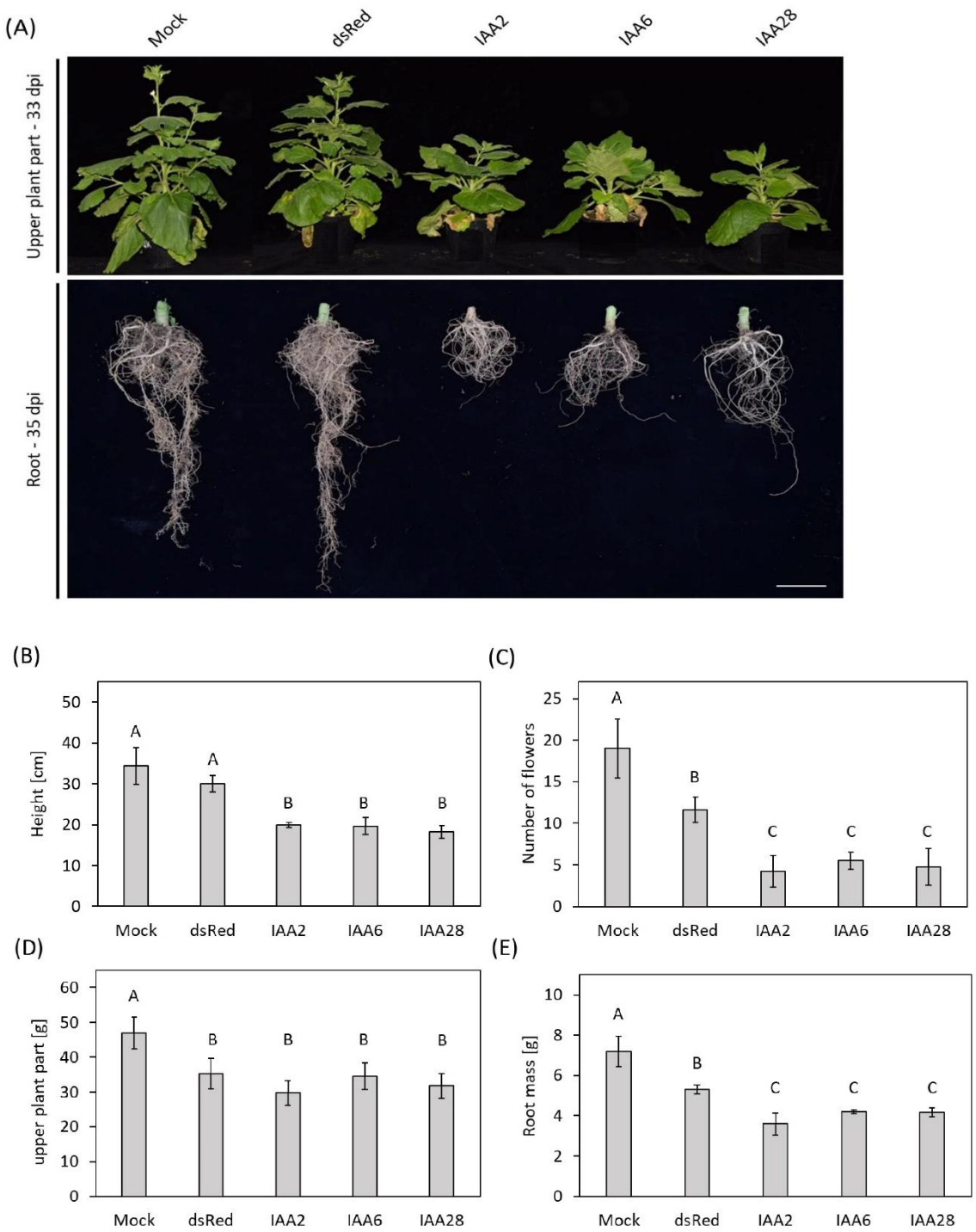

Figure 7. Heterologous expression of Aux/IAAs in *N. benthamiana*. **(A)** Upper plant part and root phenotypes of *N. benthamiana*, non-inoculated (mock), mechanically infected with TRV expressing dsRed, IAA2, IAA6, and IAA28. Pictures of the upper plant part were taken at 33 dpi and pictures of the root phenotype were taken at 35 dpi. Scale bar, 5 cm. The examinations of different plant parts are shown on the right. **(B)** plant height in cm, **(C)** number of flowers, **(D)** mass of the upper plant part in g, **(E)** mass of the root in g. Data and error bars represent the mean and the standard deviation of at least four replicates (n = 4). Significant differences are indicated as letters above the bars.

Discussion

The excessive formation of LRs is the characteristic symptom of the rhizomania disease in sugar beet. Since LR formation is controlled by auxin (Fukaki *et al.*, 2007; Lavenus *et al.*, 2013; Du & Scheres, 2018), it seems reasonable to assume that BNYVV interferes with the auxin signaling pathway for which experimental evidence was provided in the previous studies (Larson *et al.*, 2008; Gil *et al.*, 2018). In this study, we confirmed that the infection of sugar beets with BNYVV is accompanied by an increase of the auxin concentration in LRs (42 dpi) (Pollini *et al.*, 1990). Interestingly, such an effect was also observed in transgenic *A. thaliana* plants constitutively expressing p25 (Peltier *et al.*, 2011). Transcriptome analyses also revealed that the genes encoding proteins involved in auxin biosynthesis − such as tyrosine decarboxylase 1, tryptophan aminotransferase-related protein 1 and several *YUCCA* genes − are upregulated in BNYVV- infected plants (Gil *et al.*, 2020). The increased auxin content was not detected at a later stage of infection (66 dpi), which might be explained by plant compensatory mechanisms supporting auxin homeostasis, which is crucial for plant development. Whether this is a reaction of the plant, or if it is a mechanism employed by the virus to support LR formation remains unknown. However, our experiments provide a direct correlation between altered auxin content and the presence BNYVV.

The interaction of the transcriptional repressor BvIAA28 with p25 has already been described and characterized (Thiel *et al.*, 2012; Gil *et al.*, 2018). In this study, two additional p25-interacting partners were identified, namely, BvIAA2 and BvIAA6. The interaction was confirmed in Y2H, BiFC and co-IP experiments. However, the fact that only the interaction of the degradation-stable variants of the Aux/IAA proteins can be detected in co-IP experiments shows how labile the interactions are. In general, Aux/IAAs are very short-lived proteins as long as no alterations done to the protein structure to prevent their degradation (Reed, 2001). All p25-interacting Aux/IAA proteins show significant similarity to their corresponding orthologues from *A. thaliana*. BvIAA2, BvIAA6 and BvIAA28 cluster together with Arabidopsis Aux/IAA proteins involved in LR development and root hair formation (Reed, 2001; Luo *et al.*, 2018). Interestingly, the *Arabidopsis* proteins AtIAA18 and AtIAA28, which cluster together with BvIAA2, and AtIAA1 that clusters together with BvIAA6, are negative regulators (transcriptional repressors) of lateral root formation and their auxin-mediated degradation is required for proper LR development. Expression of degradation stable variants of these

proteins reduced lateral root development in *N. benthamiana* even in the presence of exogenously supplemented auxin (Fukaki *et al.*, 2002; Uehara *et al.*, 2008; Notaguchi *et al.*, 2012). Such negative regulators are also found among *Arabidopsis* proteins that cluster together with BvIAA28, namely AtIAA14/SLR and AtIAA16 (Fukaki *et al.*, 2002; Rinaldi *et al.*, 2012). However, expression of degradation stable variants of two other Aux/IAA proteins (AtIAA7/AXR2; AtIAA17/AXR3) from this cluster led to an increased number of lateral roots, indicating an enhanced auxin response (Leyser *et al.*, 1996; Nagpal *et al.*, 2000).

Heterologous expression of sugar beet Aux/IAAs using a TRV vector was employed to characterize the effect *BvIAA2*, *BvIAA6* and *BvIAA28* overexpression in *N. benthamiana*. Unfortunately, similar experiments as well as VIGS could not be performed in sugar beet because of instability of the TRV vector in sugar beet roots (see results section). Expression of either *BvIAA2*, *BvIAA6* or *BvIAA28* from TRV in *N. benthamiana* resulted in very similar phenotypes characterized by dramatic inhibition of root development confirming that the auxin-mediated regulatory pathways are highly conserved across different plant species (sugar beet versus *N. benthamiana*). Additional phenotypes associated with *BvIAA2*, *BvIAA6* or *BvIAA28* expression included a stunting and dwarfism, a significant reduction in the number of flowers, and a reduction of the root mass, as well as an overall root shortening. These effects on plant development and growth were further enhanced when a variant of BvIAA6 resistant to auxin-mediated degradation was expressed. Thus, the phenotypes closely resembled those induced by degradation-stable variants of the corresponding *Arabidopsis* homologs of sugar beet Aux/IAAs described above, *i.e*. degradation stable variants of AtIAA14/SLR, AtIAA16, AtIAA18, AtIAA19 and AtIAA28 also induced a shortening of the root accompanied by reduction in the number of lateral roots (Fukaki *et al.*, 2002; Uehara *et al.*, 2008; Notaguchi *et al.*, 2012; Rinaldi *et al.*, 2012). The expression of degradation stable AtIAA18 also caused a shortening of the internodes (Fukaki *et al.*, 2002), the phenotype that was also observed in this study, when *BvIAA2*, *BvIAA6* or *BvIAA28* were expressed from TRV vector in *N. benthamiana*. Hence, our findings that all p25 interacting Aux/IAA proteins identified so far affect root development in *N*. *benthamiana* is in agreement with the previous studies in *A. thaliana* showing that several Aux/IAAs are involved in controlling various distinct steps of root development and LR formation (Fukaki *et al.*, 2002; Knox *et al.*, 2003; Lavenus *et al.*, 2013). It is also very likely that these steps of LR development in sugar beet are controlled

by functional homologues of corresponding *Arabidopsis* Aux/IAA proteins, yet direct evidence is lacking.

Analysis of BvIAA2, BvIAA6 and BvIAA28 sequences revealed the presence of NLS signals similar to those of other Aux/IAA proteins (Abel *et al.*, 1994; Reed, 2001; Wu *et al.*, 2012; Luo *et al.*, 2018). Indeed, the subcellular localization of BvIAA2 and BvIAA6 revealed that they exclusively accumulate in the nucleus like BvIAA28 (Gil *et al.*, 2018). Moreover, the subcellular localization of BvIAA2 and BvIAA6 proteins co-expressed with p25 did not change and both proteins remained confined to the nucleus in the presence of p25. This is in contrast to the previously reported translocation of BvIAA28 into cytoplasm upon co-expression with p25 (Gil *et al.*, 2018). RT-qPCR results clearly demonstrated that the mRNA levels of *BvIAA2*, *BvIAA6* and *BvIAA28* did not show significant alterations at different stages of BNYVV infection as was tested at 28, 44 and 66 dpi. It can be speculated that p25 might exert a similar effect on BvIAA2 and BvIAA6 as the Rice dwarf virus (RDV) P2 protein on OsIAA10. RDV P2 manipulates the auxin signaling by targeting OsIAA10 in the nucleus and preventing its degradation by 26S proteasome (Jin *et al.*, 2016; Qin *et al.*, 2019). Contrary, the TMV replicase interacts with AtIAA26 and disrupts its nucleolar localization which affects the function of AtIAA26 as transcriptional repressor of auxin responsive genes (Padmanabhan *et al.*, 2005; Padmanabhan *et al.*, 2006; Padmanabhan *et al.*, 2008). This mechanism appears to be similar to that exerted by p25 on the localization of BvIAA28, which is translocated into cytoplasm in the presence of p25 (Gil *et al.*, 2018).

Attempts to identify the interaction domains in p25 and BvIAA2 and BvIAA6 yielded no results as small changes of the amino acid sequences led to a loss of interaction in either Y2H or BiFC, demonstrating the high specificity of the interaction. Even a single amino acid substitution in the NLS or NES signal of p25 disrupted the interaction. Since the expression of altered proteins used in the protein-interaction studies with p25 could be confirmed by immunoblotting we concluded that the interaction of p25 with BvIAA2 and BvIAA6 requires the full-length proteins. Viral proteins are multi-functional with an extensive networks of cellular interaction partners that has been developed during the co-evolution of viruses and their hosts (Callaway *et al.*, 2001; Nagy, 2016; Valli *et al.*, 2018). It has been observed in a previous study that sequence variation in the p25 protein affects its ability to self-interact and activate transcription in yeast one-hybrid system (Klein *et al.*, 2007). Therefore, it can be speculated

that sequence variation in p25 might affect its interaction with Aux/IAA proteins as these interactions seems to be very delicate and prone to disruption due to even slight alterations of the amino acid sequence. It was also not possible to identify interacting domains of BvIAA2 and BvIAA6 as was done for BvIAA28. BNYVV p25 appears to interact primarily with domains I and II of BvIAA28 (Gil *et al.*, 2018). By contrast, the fact that similar approaches in identification potential interacting domains in BvIAA2 and BvIAA6 were not successful is probably due to some difference in the structure of BvIAA2 and BvIAA6 proteins compared to the BvIAA28 structure. Indeed, most of the Aux/IAA proteins contain extensive intrinsically disordered regions (IDRs), which are prone to conformational changes due to interaction with other proteins (Niemeyer *et al.*, 2020). The presence of IDRs is a major factor promoting the interaction with multiple partners, thus, affecting interactions regulating stress responses, development, metabolic and signaling pathways (Covarrubias *et al.*, 2020). On one hand, IDRs can provide structural flexibility for interaction and proper positioning of Aux/IAAs on *e.g.* Cullin RING-type E3 ubiquitin ligases TIR1 (Niemeyer *et al.*, 2020). On the other hand, IDRs can be sensitive to changes of amino acid sequence when interacting with primarily ordered regions (Mishra *et al.*, 2020) and the analysis predicts that p25 is an entirely ordered protein (data not shown). In order to make more precise statements in this regard and to determine possible interaction domains, further investigations, preferably with native proteins, might shed light on the nature of these interactions.

To conclude, in addition to BvIAA28, two sugar beet Aux/IAA proteins, namely, BvIAA2 and BvIAA6, were identified in this study to interact with p25, the BNYVV virulence factor. In contrast to BvIAA28, BvIAA2 and BvIAA6 do not appear to change their subcellular localization, they are not translocated into the cytoplasm by interaction with p25 and remain confined to the nucleus. Overall, the results show that p25 sequesters negative regulators of root development and thus likely promotes LR initiation and formation. The detailed mechanism of p25 action remains to be determined, hopefully with development of appropriate genetically tractable model systems as most genetic approaches in sugar beet are still extremely challenging and time consuming.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Contribution to the Field Statement

Beet necrotic yellow vein virus (BNYVV), characterized by excessive lateral root (LR) formation is one of the most severe diseases in sugar beet cultivation. LR organogenesis is regulated by the auxin pathway, more specific several Aux/IAA transcriptional regulators. Previously, we characterized the interaction on BNYVV p25 virulence factor interacts with BvIAA28. In this work, we show that p25 also interacts with BvIAA2 and BvIAA6. Moreover, we confirmed that the BNYVV infection is, indeed, accompanied by an elevated auxin level in the infected LRs. Nevertheless, expression levels of BvIAA2 and BvIAA6 remained unchanged upon BNYVV infection. Mutational analysis indicated that interaction of p25 with either BvIAA2 or BvIAA6 requires full-length proteins as even single amino acid residue substitutions abolished the interactions. Compared to p25-BvIAA28 interaction that led to redistribution of BvIAA28 into cytoplasm, both BvIAA2 and BvIAA6 remained confined into the nucleus regardless of the presence of p25. As expected, overexpression of p25-interacting partners (BvIAA2, BvIAA6 and BvIAA28) in *Nicotiana benthamiana* induced an auxin-insensitive phenotype characterized by plant dwarfism and dramatically reduced LR development. Thus, our work reveals a distinct class of transcriptional repressors targeted by p25, and their interaction with p25 plays active role in virus virulence.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Figures

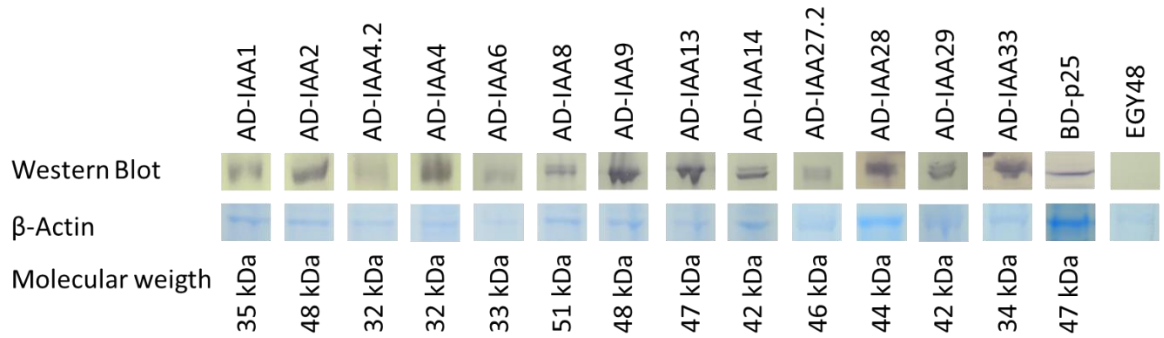

Figure S1. Expression of AD-Aux/IAA fusion proteins in yeast were detected using a HA tag and Expression of BD-p25 fusion proteins were detected using a LexA tag. Below the Western blot, β-actin (~ 43 kDa) is shown as loading control in Coomassie stained SDS gels. The molecular weight of each Aux/IAA protein is indicated below the loading controls. The yeast strain EGY48 without any plasmid served as negative control.

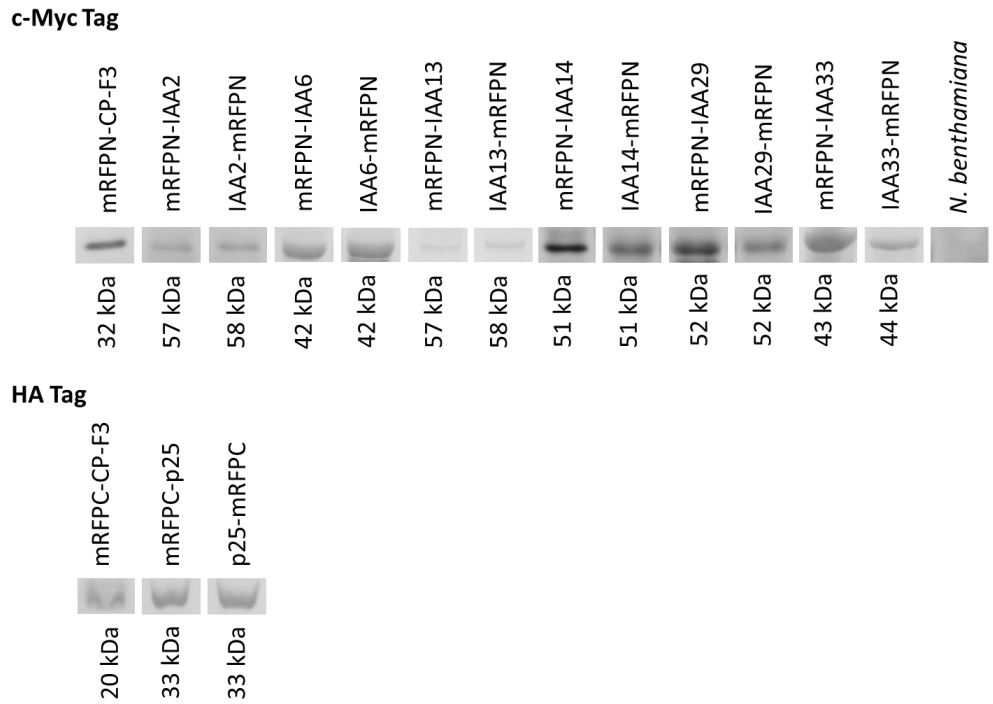

Figure S2. Detection of all fusion-proteins used in the BiFC assay by immunoblot. The name of each fusion protein, including the positive (+) and the negative (-) controls is given above each signal and the molecular weights of the proteins are given below. The upper part of the figure shows the immunoblot of C-Myc tagged fusion-proteins and the lower part shows HA tagged proteins. A protein sample from non-inoculated, healthy *N. benthamiana* leaves served as negative control to exclude unspecific binding of the antibodies. Images were taken at 4 dpi. Scale bars, 50 µm.

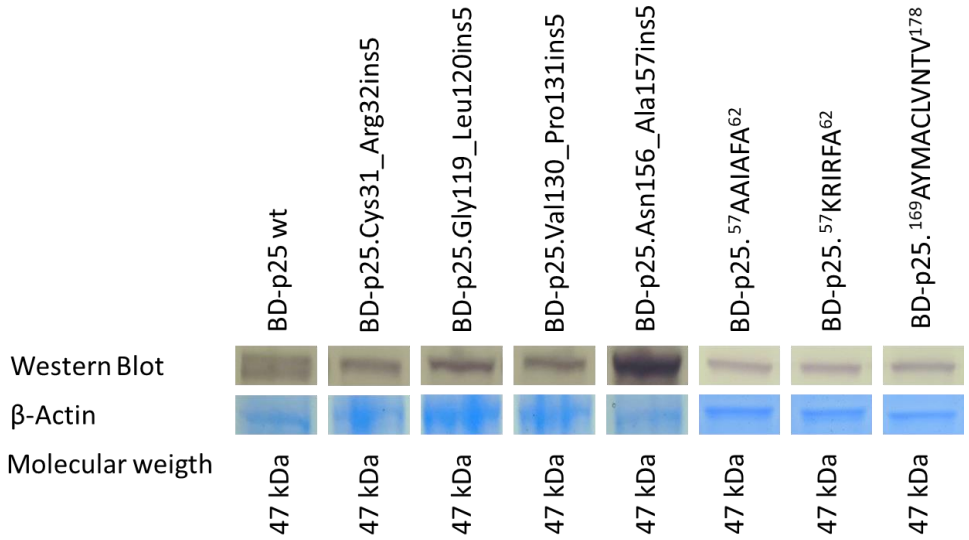

Figure S3. Detection of BD-p25 wt and different BD-p25 variants from the yeast experiments by immunoblot. The first four p25 fusion proteins variants after p25 wt were obtained from the Pentapeptide scanning mutagenesis and the last three fusion protein variants represent p25 varieties with mutated NLS and NES motifs. As in the other experiment fusion proteins with the BD were detected using a LexA. Below the Western blot, β-actin (~ 43 kDa) is shown as loading control in Coomassie stained SDS gels. The molecular weight of each p25 protein variant is indicated below the loading controls.

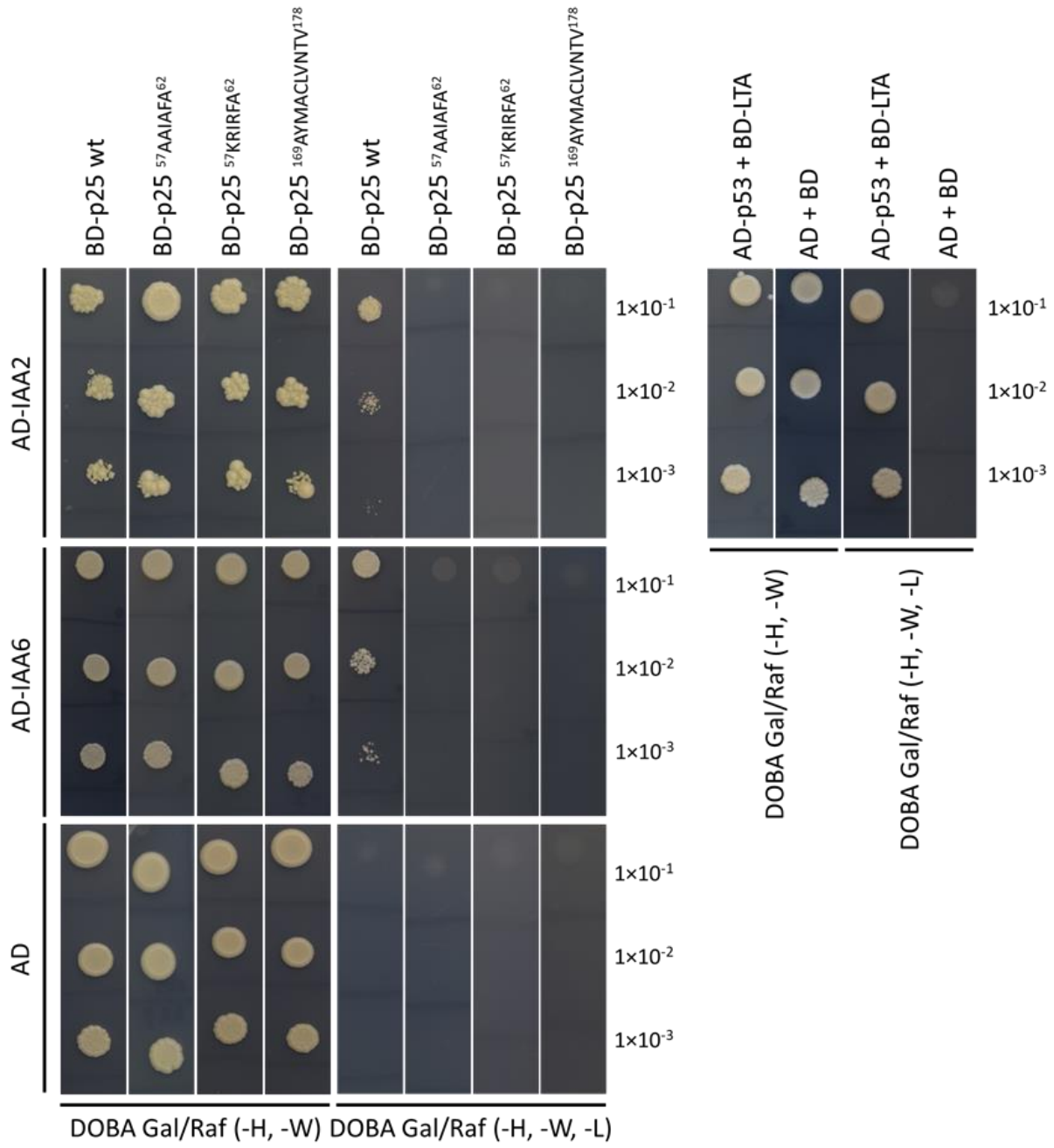

Figure S4. Results from a YTH experiment where IAA2 and IAA6 were tested for interaction with four BNYVV p25 varieties, p25 wt, p25 ^{57}AAIAFA^{62}, p25 ^{57}KRIRFA^{62} and p25 ^{169}AYMACLVNTV^{178}. The positive control AD-p53 with BD-LTA and the negative control AD(-empty) with BD(-empty) were supplied by MoBiTech. BNYVV p25 was fused to the BD and the IAAs to the AD to test for interaction. Yeast transformants, containing both plasmids were selected on DOBA Glu (-H, -W), single colonies were resuspended in water and diluted 1×10^{-1}- 1×10^{-3}. 5 µl of each dilution was spotted on the control medium (DOBA Glu (-H, -W) and selection medium (DOBA Gal/Raf (-H, -W, -L)). AD without any fusion proteins and transformed with all BD-p25 varieties, served as control for autoactivation. AD - activating domain; BD - binding domain; DOBA – Dropout Base Agar.

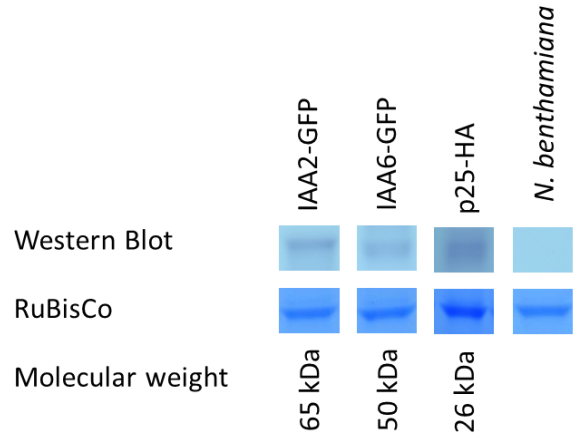

Figure S5. Detection of IAA2-GFP, IAA6-GFP and p25-HA by immunoblot. All fusion proteins were detected by HA antibodies. Tobacco Ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCo) served as loading control) in Coomassie stained SDS gels (~ 55 kDa). The molecular weight of each Aux/IAA protein is indicated below the loading controls. A protein sample from non-inoculated, healthy *N. benthamiana* leaves served as negative control to exclude unspecific binding of the antibodies.

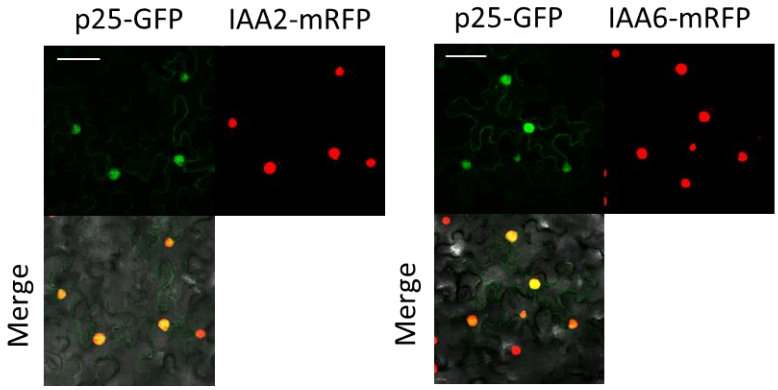

Figure S6. Subcellular localization of p25 fused to GFP (p25-GFP) co-expressed with the interacting Aux/IAAs fused to mRFP (IAA2-mRFP and IAA6-mRFP) in *N. benthamiana* epidermal leaf cells. Images were taken at 4 dpi. Scale bars, 50 µm.

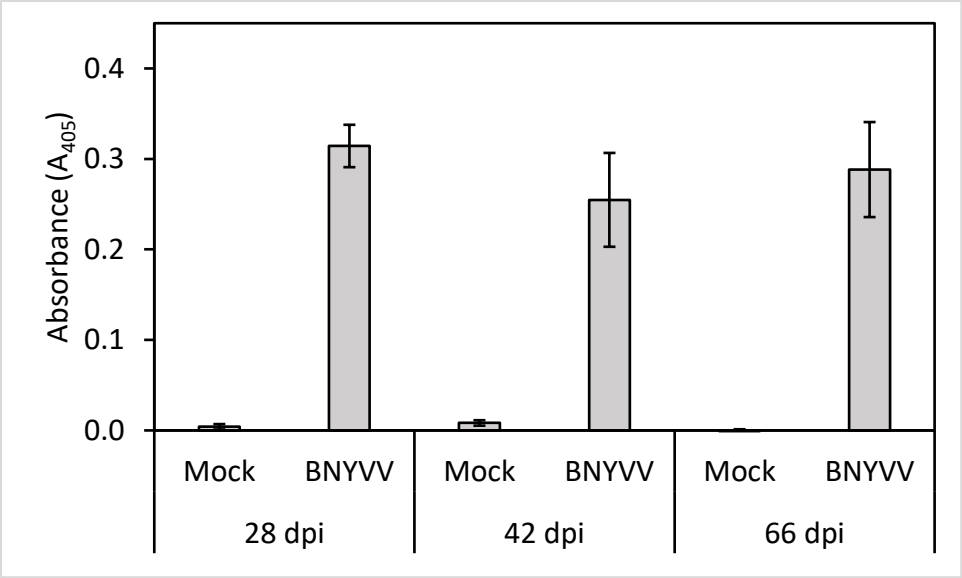

Figure S7. Mean absorbance values (A_{405}) determined by double antibody sandwich ELISA in lateral roots of BNYVV inoculated and non-inoculated (mock) sugar beets used for qPCR quantification of *IAA2*, *IAA6* and *IAA28*. The plants were harvested after 28, 42 and 66 dpi. Vertical bars indicate SD (n=5).

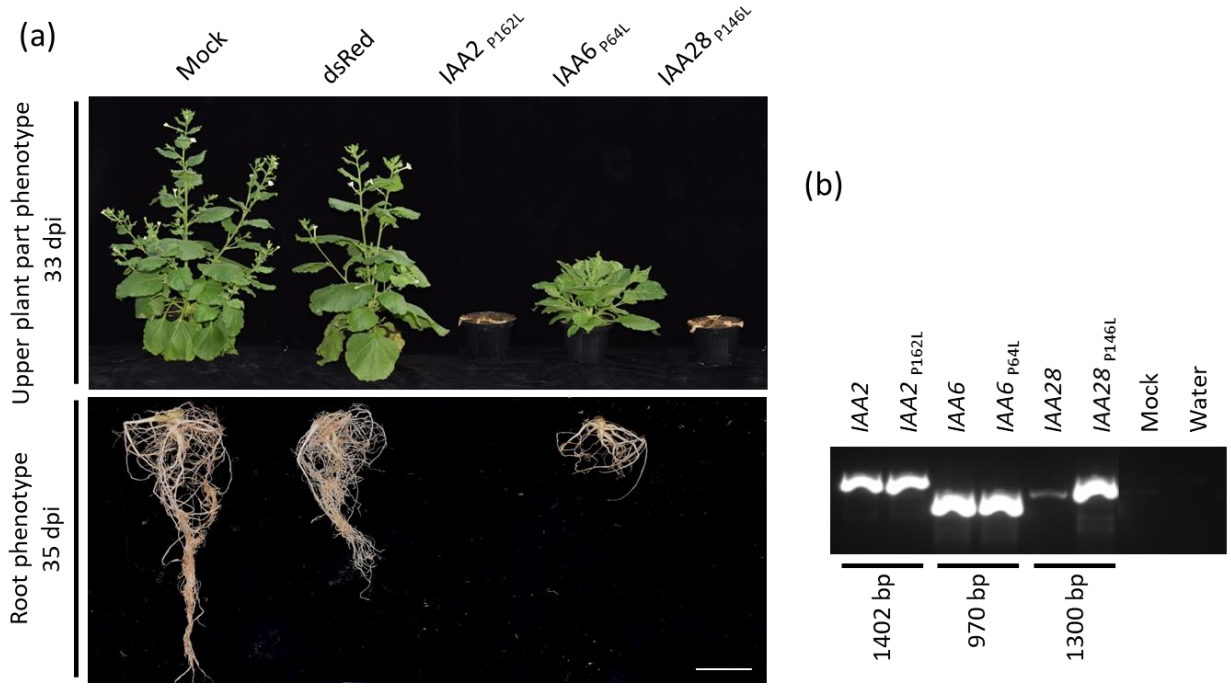

Figure S8. (A) Upper plant part and root phenotypes of *N. benthamiana*, non-inoculated (mock), infected with TRV expressing dsRed and infected the three TRV mutants overexpressing the degradation resistant Aux/IAA variants IAA2 $_{P162L}$, IAA6 $_{P64L}$ and IAA28 $_{P146L}$. Pictures of the upper plant part were taken at 33 dpi, pictures of the root phenotype were taken at 35 dpi. Scale bar, 5 cm. **(B)** PCR amplification of all *Aux/IAA* genes in cDNA samples made from TRV systemically infected *N. benthamiana* leaf samples. The names of the genes are given above the signals and the size of the signals is given below the picture in base pairs (pb). cDNA from a non-inoculated *N. benthamiana* plant served as negative control (mock).

Tables

Table S1. List of all *IAA* genes from sugar beet

Gene	KEGG Acc. No.	Oligonucleotide pair	Size (bp)
IAA1	104904635	#9 + #10	618
IAA2	104883127	#11 + #12	996
IAA4	104890935	#13 + #14	546
IAA4.2	104906976	#15 + #16	534
IAA6	104904637	#17 + #18	564
IAA8	104883520	#19 + #20	1098
IAA9	104897812	#21 + #22	1020
IAA13	104899391	#23 + #24	978
IAA14	104894592	#25 + #26	837
IAA27.2	104904711	#29 + #30	945
IAA29	104901993	#31 + #32	795
IAA32	104884870	#33 + #34	594
IAA33	104902411	#35 + #36	603

Table S2. List of all oligonucleotides used in this study. The Oligonucleotides are sorted according to their application (YTH, BiFC, qPCR, co-localization, and TRV-expression).

Primer Name	Sequence (5' to 3')
YTH	
#1 IAA28F	CTAGAATTCATGTTGAGTGCTGAGATTAGAGACACTTATAGCAC
#2 IAA28R	CAGTCTCGAGTCAGCTTCTACTCTTGCATTTCTCGACAGC
#3 p25 F	ATCGAATTCATGGGTGATATATTAGGCGC
#4 p25 Atyp	TACCTCGAGCTAATCATCATCATCAACAC
#6 BAIT seq.	CGTCAGCAGAGCTTCACC
#7 PREY seq.	CTGAGTGGAGATGCCTCC
#9 IAA1F	CTAGAATTCATGGAACAACAACAAGAAGT
#10 IAA1R	TACCTCGAGGTTACTCAATGTTGGATGGTG
#11 IAA2F	CTAGAATTCATGGGTGAAAGTAACCCAAA

#12 IAA2R	TACCTCGAGTCACTTGGATGCACTCTC
#13 IAA4F	CTAGAATTCATGGAGATGAACAAGAAAGAAA
#14 IAA4R	TACCTCGAGTTAAGCCAAACAACCCAAG
#15 IAA4.2F	CTAGAATTCATGTATAGGAAAGAAGATGATCAA
#16 IAA4.2R	TACCTCGAGTTAGCATTTCTCCAAAGCAG
#17 IAA6F	CTAGAATTCATGTCGAAAGCGGGTT
#18 IAA6R	TACCTCGAGTCACCCATGGCATTGC
#19 IAA8F	CTAGAATTCATGTCTGGTGTTAGAGAGGA
#20 IAA8R	TACCTCGAGCTAGCTCCTGTTCCTGC
#21 IAA9F	CTAGAATTCATGTCTCCCCCATTATTGG
#22 IAA9R	TACCTCGAGCTAGTTCCGGCTCTTAGAT
#23 IAA13F	CTAGAATTCATGGAAGCTGTAATGGGG
#24 IAA13R	TACCTCGAGTTATATAGGCCGACTTCTTTG
#25 IAA14F	CTAGAATTCATGGAAGTTGGGTTGATGAA
#26 IAA14R	TACCTCGAGTTAGCTCCTGTTCTTGCA
#27.2 IAA27F.2	CATGTCAATGGGTTTTGAAGA
#28.2 IAA27R	TACCTCGAGTTAGCCATCGCTAACTCTTG
#29 IAA27.2F	CTAGAATTCATGTCTAGGCCATTAGAACA
#30 IAA27.2R	TACCTCGAGTCAGGCTTCAGTCTTACAC
#31 IAA29F	CTAGAATTCATGGAGCTTGAATTAGGTCT
#32 IAA29R	TACCTCGAGTTAATCATCCCTTCTCCTTAGC
#33 IAA32F	CTAGAATTCATGGAATCGAACATGGCA
#34 IAA32R	TACCTCGAGGCAAAGAGGTTAAGGATTGT
#35 IAA33F	CTAGAATTCATGTATAACAACATGAATAACAATAAGA
#36 IAA33R	TACCTCGAGCTAGTGTTTTGTGCTCCTTT
#39 pJG-Ins. seq. up	GACTGGCTGAAATCGAATGG
#40 pJG-Ins. seq. Low	GCCGACAACCTTGATTG
#280_pJG4-5Domain_fw	CTCGAGAAGCTTTGGACTTC
#281_IAA2DI+II_rv	TCAACTCGGAATCGCACTC
#282_IAA2_DIII+IV-fw	AAGCCTGTGAATGAAAAATCAG
#284_IAA6DI+II_rv	TCACTCATTACCAATGCTCCTCC
#285_IAA6_DIII+IV-fw	AAGGAATGTATTGAGGCATCAAAG
#293_IAA6DII-IV_fw_new	GATATAGTTGGCGGCCAC

#299_IAA6YTH_DI-III-fw	TGACTCGAGAAGCTTTGGAC

BiFC

#44 pCB-mRFP-Nterm-fw	CGATCCTCTAGAGTCCGCAAAAATCACC
#45 pCB-mRFP-Nterm-rv	CTCCACCAGATCCACCTCCGG
#46 IAA28Rz2-N-fw	GATCTGGTGGAGGTGGATCCAGCAGCACGATTAATTTCGAAGAGACAGA
#47 IAA28Rz2-N-rv	CTCTAGAGGATCGATCCTTAGCTTCTACTCTTGCATTTCTCGACAGC
#48 mRFP-N-fw	CTACAAGACCGACATCAAGCTGGAC
#49 mRFP-N-rv	CGAAACCCTATAAGAACCCTAATTCCCT
#50 pCB-mRFP-Cterm-fw	GGAGGTGGATCTGGTGGAGGTAC
#51 pCB-mRFP-Cterm-rv	GTGCTGCTTGTTATATCTCCTTCGAAGATCT
#52 IAA28Rz2-C-fw	TCTTCGAAGGAGATATAACA ATG AGCAGCACGATTAATTTCGAAGAGAC
#53 IAA28Rz2-C-rv	CCTCCACCAGATCCACCTCCGCTTCTACTCTTGCATTTCTCGACAG
#54 mRFP-C-fw	TTCTCAACACAACATATACAAAACAAACGAATC
#55 mRFP-C-rv	GGAGCCCTCCATGCGC
#56 pCB-smRSGFP-Nterm-fw	TGAGTCCGCAAAAATCACCAGTCTCTC
#57 pCB-smRSGFP-Nterm-rv	ACCTCCACCAGATCCACCTCCTTTGTAT
#58 p25-N-fw	GAGGTGGATCTGGTGGAGGTATGGGTGATATATTAGGCGCAG
#59 p25 ATyp-N-rv	TGGTGATTTTTGCGGACTCAACCATCATCATCAACACCGTC
#60 smRSGFP-N-fw	CACAATCTGCCCTTTCGAAAGATCC
#61 smRSGFP-N-rv	CCCTAATTCCCTTATCTGGGAACTAC
#62 pCB-smRSGFP-Cterm-fw	GGAGGTGGATCTGGTGGAGG
#63 pCB-smRSGFP-Cterm-rv	TGTTATATCTCCTTCGAAGATCTATCG
#64 p25-C-fw	TCTTCGAAGGAGATATAACAATGGGTGATATATTAGGCGCAG
#65 p25 A-Typ-C-rv	CCTCCACCAGATCCACCTCCACCATCATCATCAACACCGTC
#66 smRSGFP-C-fw	GAAAATTTGTGCCCATTAACATCACC
#67 smRSGFP-C-rv	CAATCCCACTATCCTTCGCAAGACC
#69 Seq. pBIN19 fw	CAAAAGTTGATTTCTGAGGAGGATCTTGGT
#70 Seq. pBIN19 rev	AAATTTTATTGATAGAAGTATT
#71 pCB-mRFPN-Nterm-fw	GGATCCACCTCCACCAGATCCACC
#72 pCB-mRFPN-Nterm-rv	TAAGGATCGATCCTCTAGAGTCCGCAAAAAT
#73 pCB IAA28sus.N-fw	GATCTGGTGGAGGTGGATCCATGTTGAGTGCTGAGATTAGAGACACTTAT
#74 pCB IAA28sus.-N-rv	CTCTAGAGGATCGATCCTTA TCAGCTTCTACTCTTGCATTTCTCGACAGC

#75 pCB mRFPN-N-fw_seq	CTACAAGACCGACATCAAGCTGGAC
#76 pCB mRFPN-N-rv_seq	ACATGAGCGAAACCCTATAAGAACCC
#77 pCB-mRFPN-Cterm-fw	TAAGGATCGATCCTCTAGAGTCCGC
#78 pCB-mRFPN-Cterm-rv	GGATCCACCTCCACCAGATCCA
#79 pCB IAA28sus-C-fw	TCTTCGAAGGAGATATAACAATGTTGAGTGCTGAGATTAGAGACACTTATAGCAC
#80 pCB IAA28sus-C-fv	CCTCCACCAGATCCACCTCCGCTTCTACTCTTGCATTTCTCGACAGC
#81 pCB mRFPN-C-fw_seq	CTACAAGACCGACATCAAGCTGGAC
#82 pCB mRFPN-C-rv_seq	ACATGAGCGAAACCCTATAAGAACCC
#83 pCB IAA28 Rz2 (-10AS)N-fw	GATCTGGTGGAGGTGGATCCAGCAGCACGATTAATTTCGAAGAGACAGA
#84IAA28 Rz2 (-10AS)-C-fw	TCTTCGAAGGAGATATAACAAGCAGCACGATTAATTTCGAAGAGACAGA
#85 pCB-mRFPC-Nterm-fw	TAAGGATCGATCCTCTAGAGTCCGC
#86 pCB-mRFPC-Nterm-rv	GGATCCACCTCCACCAGATCC
#87 pCB p25 BTyp -N-fw	GATCTGGTGGAGGTGGATCCATGGGTGATATATTAGGCGCAGTTTAT
#88pCB p25 BTyp -N-rv	CTCTAGAGGATCGATCCTTACTAATCATCATCATCAACACCGTCAGG
#89 pCB p25 BTyp-C-fw	TCTTCGAAGGAGATATAACAATGGGTGATATATTAGGCGCAGTTT
#90 pCB p25 BTyp-C-rv	CCTCCACCAGATCCACCTCCATCATCATCATCAACACCGTCAGG
#91 pCB mRFPC-C-fw_seq	ACATGAGCGAAACCCTATAAGAACCC
#110 IAA28sus. F BiFC	TGAGGATCCATGTTGAGTGCTGAGATTAGAGACAC
#111 IAA28sus.+Rz2(10AS) R (stopp) BiFC	CTAGTCGACTCAGCTTCTACTCTTGCATTTCTC
#112 IAA28sus.+Rz2(10AS) R BiFC	CTAGTCGACGCTTCTACTCTTGCATTTCTCGAC
#114 pCB mRFPN GOI Seq.	CTCCACCGAGCGGATGTAC
#115 pCB GOI mRFPN Seq.	CTCAAGCAATCAAGCATTCTAC
#134 IAA2 F BiFC	TGAGGATCCATGGGTGAAAGTAACCCAAA
#135 IAA2 R Stopp BiFC	CTAGTCGACCTTGGATGCACTCTCCAC
#136 IAA2 R BiFC	CTAGTCGACTCACTTGGATGCACTCTC
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#213mRFPN-GOIrv	GGATCCACCTCCACCAG
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#287_ pBiFC_Domain_rv	GGATCCACCTCCACCA
#294_pBiFC_IAA6DI,III+IV_rv	GCCAACTATATCGGAAAACACC
#295_pBiFC_IAA6DI+II,IV_fw	GAGGCATTGAAAGATGCG
#296_pBiFC_IAA6DI+II,IV_rv	CTTTGATGCCTCAATACATTCC
#297_pBiFC_IAA6DI-III _rv	CGCATCTTTCAATGCCTC
#298_ pBiFC_IAA6DI-III_fw	TGAGTCGACTAAGGATCGATC
#316_mRFPC-p25-rev	GACTCTAGAGGATCGATCCTTAACCATCATCATCAACACCGTC
#298_ pBiFC_IAA6DI-III_fw	TGAGTCGACTAAGGATCGATC
#316_mRFPC-p25-rev	GACTCTAGAGGATCGATCCTTAACCATCATCATCAACACCGTC
qPCR	
#118 IAA2 qPCR fw.	CACAGCCCTGTTGCACTAGA
#119 IAA2 qPCR rev.	AATTGGAGGCCAACCCACAA
#120 IAA6 qPCR fw.	GCATGGATGGTGTGCCTTTC
#121 IAA6 qPCR rev.	CCGCATCTTTCAATGCCTCG
#348_GAPDHqPCR	CACCACCGATTACATGACATACA
#349_GAPDHqPCR7R	GGATCTCCTCTGGGTTCCTG
#350_EF1AlphaqPCR7F	GCTTTTGAGGATCTCTGGCG
#351_EF1Alpha qPCR7R	AAGCCTTAGAGTCAGCTGCT
co-localization	
#218pCBmRFP-GOIfw.	ATGGTCGACTAAGGATCGATCCTCTAGAGTC
#219pCBmRFP-GOIrv.	GTAGGATCCACCTCCACCAGATCCAC

#221pCB GOI-mRFP fw.	ATGGTCGACGGAGGTGGATCTGGTGG
#222pCB GOI-mRFP rv.	GTAGGATCCTGTTATATCTCCTTCGAAGATCTATC
#304_p25-pCB_rv	TGATTTTTGCGGACTCTAGATTAACCATCATCATCAACACCG
#305_GSlink-p25_fw	GAGGTGGATCTGGTGGAGGTATGGGTGATATATTAGGCGCAG
#306_p25-GS-link_rv	CCTCCACCAGATCCACCTCCACCATCATCATCAACACCG
#307_IAA2-GS-link_rv	CCTCCACCAGATCCACCTCCCTTGGATGCACTCTCCACCA
#308_GOI-pCB-fw	TGAGTCCGCAAAAATCACC
#309_IAA2_pCB_rv	TGGTGATTTTTGCGGACTCACTTGGATGCACTCTCCACC
#310_IAA6_pCB_rv	TGGTGATTTTTGCGGACTCACCCATGGCATTGCTTC
#314_HA-35s	CTGACTATGCGTGATTCTCCAGAATAATGTGTGAG
#315_GS-HA	GAACATCGTATGGGTAACCTCCACCAGATCCAC
#317_pCBfwds	GACGGCCACTACGACGC
#318_HArev	AGCGTAATCTGGAACATCGTATGG
#319_dsRed-pCB_fw	ACGATGTTCCAGATTACGCTATGGTGCGCTCCTCCAAG
#320_HA-dsRed_rv	TCGGCGTCGTAGTGGCCGTCTTACAGGAACAGGTGGTGGCG
#321_dsRedGS_rv	CCTCCACCAGATCCACCTCCCAGGAACAGGTGGTGGC
#322_GFP-SV40_fw	AAGAAAGGTTTGATTCTCCAGAATAATGTG
#323- GFP-SV40-rv	TTCTTTTTTGGGTACAGCTCGTCCATG
TRV	
AUX2_fw	CTTACCCGAGTTAACGAGCCATGGGTGAAAGTAACCCAAAATTG
AUX2_rv	CTCGGTACCGAGCTCGAATTCTACTTGGATGCACTCTCCACCA
AUX6_fw	CTTACCCGAGTTAACGAGCCATGTCGAAAGCGGGTTTCGAAC
AUX6_rv	CTCGGTACCGAGCTCGAATTCTACCCATGGCATTGCTTCTTTGACTG
AUX28_fw	CTTACCCGAGTTAACGAGCCATGTTGAGTGCTGAGATTAGA
AUX28_rv	CTCGGTACCGAGCTCGAATTCTAGCTTCTACTCTTGCATTTCTC
AUX2_L162P-fw	TTCCAATTCGATCGTTCCGAAAG
AUX2_L162P-rv	GCCAACCCACAACTGGAGTAG
AUX6_L64P-fw	TAGGAGGAGGAGCATTGGTAATG
AUX6_L64P-rv	TATGAGCACACTGGAAGCCAC
AUX28_L146P-fw	CCAGTTCGAGCATTCAGGAAAC
AUX28_L146P-rv	TAGCCAACCTACGACTTGTGC

Manuscript III

Comparative analysis of virus pathogenicity and resistance-breaking between the P- and A-type from the beet necrotic yellow vein virus using infectious cDNA clones

Maximilian Müllender[1], Edgar Maiss[2], Mark Varrelmann[1], Sebastian Liebe[1*]

[1]Institute of Sugar Beet Research, Department of Phytopathology, Göttingen, Germany

[2]Institute of Horticultural Production Systems, Plant Virology, Department of Phytomedicine, Leibniz University, Hannover, Germany

***Correspondence:** Liebe, Sebastian <liebe@ifz-goettingen.de>

Keywords: beet necrotic yellow vein virus, *Beta vulgaris*, *Rz1*, *Rz2*, plant virus, P-type, resistance-breaking, virus evolution

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Repositories

BNYVV P-type Acc. No.:

Beet necrotic yellow vein virus segment RNA1, complete sequence - Accession: MZ836262.1

Beet necrotic yellow vein virus segment RNA2, complete sequence - Accession: MZ836263.1

Beet necrotic yellow vein virus segment RNA3, complete sequence - Accession: MZ836264.1

Beet necrotic yellow vein virus segment RNA4, complete sequence - Accession: MZ836265.1

Beet necrotic yellow vein virus segment RNA5, complete sequence - Accession: MZ836266.1

Abstract

The A-type of the beet necrotic yellow vein virus (BNYVV) is widely distributed in Europe and one of the major virus types causing rhizomania disease in sugar beet. The closely related P-type is mainly limited to a small region in France (Pithiviers). Both virus types possess four RNAs (RNA1-4), but the P-type harbors an additional fifth RNA species (RNA5). The P-type is associated with stronger disease symptoms and resistance-breaking of *Rz1*, one of the two resistance genes which are used to control BNYVV infection. These characteristics are presumably due to the presence of RNA5, but experimental evidence is missing. We generated the first infectious cDNA clone of BNYVV P-type to study its pathogenicity in sugar beet in comparison to a previously developed A-type clone. Using this tool, we confirmed the pathogenicity of the P-type clone in the experimental host *Nicotiana benthamiana* and the two *Beta* species *B. macrocarpa* and *B. vulgaris.* Independent of RNA5*,* both the A- and P-type accumulated in lateral roots and reduced the taproot weight of a susceptible sugar beet genotype to a similar extent. In contrast, only the P-type clone was able to accumulate a virus titer in an *Rz1* resistant variety whereas the A-type clone failed to infect this variety. The efficiency of the P-type to overcome *Rz1* resistance was strongly associated with the presence of RNA5. Only a double resistant variety, harboring *Rz1* and *Rz2* prevented an infection with the P-type. Reassortment experiments between the P- and A-type clones demonstrated that both virus types can exchange whole RNA components without losing the ability to replicate and to move systemically in sugar beet. Although our study highlights the close evolutionary relationship between both virus types, we could demonstrate distinct pathogenicity properties that are attributed to the presence of the RNA5 in the P-type.

Impact statement

The biological function of RNA5 in P-type populations of BNYVV is still unknown since no infectious cDNA clone is available. Here, we developed the first infectious cDNA clone of the BNYVV P-type to elucidate the role of the RNA5 in pathogenicity and resistance breaking. Together with our previously developed A-type clone, we were able to compare both virus types in sugar beet. Our results revealed no differences in symptom severity and virus accumulation, but both virus types differed in their ability to overcome the major resistance gene *Rz1*. The resistance-breaking ability of the P-type was mediated by the RNA5 which is absent in the A-type. We could also show that reassortments from both virus types are able to replicate and move systemically in sugar beet. This strongly underlines the close evolutionary relationship between both virus types. Our results provide also the first experimental evidence for a specific role of the RNA5 in resistance-breaking by the P-type which extends our knowledge on BNYVV. It also demonstrates the adaptability of viral genomes towards plant resistance traits. The availability of two infectious BNYVV clones now allows a detailed study of the interaction between both virus types.

Data summary

The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files.

Introduction

Beet necrotic yellow vein virus (BNYVV) is the causal agent of rhizomania disease in sugar beet, which is characterized by a reduced size of the taproot with massive lateral root development and yellowing along the leaf veins (Tamada *et al.*, 1989; Tamada & Abe, 1989). BNYVV belongs to the *Benyviruses* within the family *Benyviridae* (Gilmer *et al.*, 2017) and is transmitted by *Polymyxa betae,* an obligate intracellular parasite of sugar beet lateral roots (Tamada & Kondo, 2013). Resting spores containing infectious virus particles can survive in the soil for decades. The genome of BNYVV consists of four to five positive-sense, single stranded RNAs. RNA1 possesses one open reading frame (ORF) encoding motifs for a methyltransferase, a helicase, a papain-like protease and an RNA-dependent RNA polymerase (Bouzoubaa *et al.*, 1987; Richards & Tamada, 1992). RNA2 possesses six ORFs, encoding a coat protein (CP), terminated by a suppressible UAG stop codon, a CP-read-through (CP-RT) protein, a triple

gene block (TGB) for viral cell-to-cell movement and a small 14 kDa cysteine-rich protein, responsible for viral suppression of RNA silencing (VSR) (Tamada & Kusume, 1991; Chiba *et al.*, 2013). The pathogenicity factor p25 encoded on RNA3 is important for symptom development in *Beta* species (Tamada *et al.*, 1989; Koenig *et al.*, 1991; Lauber *et al.*, 1998). Additionally, a truncated non-coding RNA3 (ncRNA3) produced by 5'→3' Xrn Exoribonuclease activity is responsible for systemic movement in *Beta* species (Lauber *et al.*, 1998; Flobinus *et al.*, 2018). RNA4 is mainly involved in efficient vector transmission, symptom development and silencing suppression in roots (Tamada & Abe, 1989; Rahim *et al.*, 2007; Wu *et al.*, 2014). Certain BNYVV isolates harbor an additional RNA5 which encodes a second pathogenicity factor (p26) (Koenig *et al.*, 1997).

The control of BNYVV relies solely on the cultivation of resistant varieties that avoid high yield losses under disease pressure. *Rz1* is the major resistance gene that is used since several decades in all sugar beet varieties (Lewellen *et al.*, 1987). Later, a second resistance gene (*Rz2*) was identified that appears to be more effective against BNYVV than *Rz1* and is based on a different resistance mechanism (Scholten *et al.*, 1994; Scholten *et al.*, 1996; Scholten *et al.*, 1999). Although there are some varieties available carrying both resistance genes, *Rz1* is currently the major resistance source in commercial varieties. This has led to a strong selection pressure on the virus population and favored the development of resistance-breaking isolates. Until now, such isolates have been reported from countries in Asia, Europe and the US (Liu *et al.*, 2005; Liu & Lewellen, 2007; Pferdmenges *et al.*, 2008; Bornemann *et al.*, 2015; Galein *et al.*, 2018; Yilmaz *et al.*, 2018; Weiland *et al.*, 2019). Comparative analysis revealed a high sequence variability at the amino acid (aa) positions 67-70 (tetrad) in the pathogenicity factor p25 (Schirmer *et al.*, 2005; Acosta-Leal *et al.*, 2010; Chiba *et al.*, 2011). Specific aa variants were only found in resistance-breaking isolates. Moreover, it could be demonstrated by means of reverse genetics that a single mutation at aa 67 from alanine to valine already mediates *Rz1* resistance-breaking (Koenig *et al.*, 2009b; Liebe *et al.*, 2020). Until now, *Rz1* resistance-breaking mediated by mutations of the tetrad in p25 has been demonstrated so far only for the A-type of BNYVV (Liebe *et al.*, 2020).

In general, BNYVV can be divided into three virus types based on the CP sequence, namely A-, B- and P-type (Kruse *et al.*, 1994; Koenig *et al.*, 1997). The A- and B-type are the major virus types displaying a worldwide distribution (Schirmer *et al.*, 2005). The P-type is closely related

to the A-type but displays a minor distribution (Miyanishi *et al.*, 1999). After the first identification in a small area in France (Pithiviers), the P-type was found later also in Kazakhstan, UK and Iran (Koenig *et al.*, 1997; Koenig & Lennefors, 2000; Ward *et al.*, 2007; Mehrvar *et al.*, 2009). Despite their clear distinction, mixed infections with A-, B- and P-type have been reported in the past (Galein *et al.*, 2018). The genome of the P-type possesses an additional RNA5 that is absent in European A- or B-type isolates. Only some A- and B-type isolates from Asia also carry an additional RNA5 (J-type), but this RNA is phylogenetically distinct from the P-type RNA5 (Miyanishi *et al.*, 1999). Both RNA5 types encode the pathogenicity factor p26 (Koenig *et al.*, 1997) whereby the exact role of p26 in viral pathogenicity is not clear yet. A recent study demonstrated that RNA5 (J-type) from Asian A-type isolates is responsible for enhanced symptom development and *Rz1* resistance breaking (Tamada *et al.*, 2020). Therefore, it is likely that the P-type RNA5 has similar properties, but this hypothesis requires experimental prove.

Previous studies showed that natural populations from the P-type are also able to overcome *Rz1* resistance (Pferdmenges *et al.*, 2008; Koenig *et al.*, 2009a; Bornemann & Varrelmann, 2011; Bornemann *et al.*, 2015). Interestingly, P-type isolates display no variability in the tetrad of p25 as observed for the A-type. Therefore, it is hypothesized that the RNA5 in the P-type is responsible for resistance-breaking rather than mutations in the tetrad of p25. Apart from that, there is evidence that the P-type is more aggressive than the closely related A-type (Heijbroek *et al.*, 1999). However, previous studies addressing the biological significance and pathogenicity of the P-type are based on natural infection using infested soil (Pferdmenges *et al.*, 2008; Koenig *et al.*, 2009a; Tamada *et al.*, 2020). BNYVV infested soil can harbor different BNYVV virus types, multiple tetrads as well as other soil-borne pathogens. Such problems can be avoided when a reverse genetic system is applied, even if it doesn't resemble natural transmission. We have shown in a previous study that an infectious cDNA clone of the BNYVV A-type can induce a rhizomania infection in sugar beet without natural vector based inoculation (Liebe *et al.*, 2020). In this study, we generated the first infectious cDNA clone of the BNYVV P-type. Together with our A-type clone, we studied the pathogenicity of both virus types in sugar beet with particular focus on *Rz1*-resistance breaking. Furthermore, we investigated the infectivity of RNA1-3 reassortments between both virus types. Our results

highlight the close relationship between both virus types but also underlines distinct pathogenicity properties regarding *Rz1* resistance-breaking.

Methods

Generation of an infectious BNYVV P-type cDNA clone

BNYVV P-type viral RNA was isolated from soil, naturally infested with a BYNVV P-type population, collected in France (Pithivier) (Wetzel *et al.*, 2021). For RNA isolation and amplification of cDNAs, susceptible sugar beet plants were grown in the infested soil as described by Wetzel *et al*., 2021 (Wetzel *et al.*, 2021). Lateral roots (100–150 mg) were harvested for RNA extraction using the NucleoSpin RNA Plant kit (Macherey-Nagel) according to the manufacturer's instructions. Homogenization of the root material was performed in extraction buffer for 45 s at 5,000 rpm using the Precellys 24 tissue homogenizer (Bertin Technologies SAS). RevertAid H Minus reverse transcriptase (ThermoFisher) and primers shown in Table S1 were used for reverse transcription of approx. 500 ng RNA into cDNA. Subsequently several PCRs with primers (Table S1) specific for RNA1-5 as well as with primers containing overhangs for the vector pDIVA (Acc. No. KX665539) (Laufer *et al.*, 2018b) were conducted using the Phusion Flash High-Fidelity PCR Master Mix (ThermoFisher). To obtain the five plasmids for the infectious cDNA clone, amplified DNA fragments were either treated with SureClean (Bioline) or purified from agarose gels using the NucleoSpin Gel and PCR Clean-up kit according to the manufacturer's instructions (Macherey-Nagel), followed by Gibson assembly (Gibson *et al.*, 2009) into PCR amplified pDIVA. Briefly, 5-13 subclones were established for RNA 1-3, reamplified and supplemented with PCR fragments of the missing parts of each RNA. Plasmids containing RNA4 and RNA5 were established in a single Gibson assembly step. According to the cloning strategy a poly-A tail was added in a final step to plasmids containing RNA1-5. The generated plasmids were transformed into chemically competent NM522 *Escherichia coli* cells and BNYVV sequences of the five viral RNAs were verified by commercial capillary Sanger sequencing (Microsynth Seqlab). The full-length sequences of all five BNYVV P-type RNAs were deposited in the database of the National Center for Biotechnology Information (NCBI) as follows: RNA1: MZ836262.1, RNA2: MZ836263.1, RNA3: MZ836264.1, RNA4: MZ836265.1, RNA5: MZ836266.1.

Virus Inoculation

Electrocompetent cells of the *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*/ *Agrobacterium fabrum*) strain C58/C1 (Voinnet *et al.*, 1998) were used for transformation of the BNYVV A-type infectious clone comprising RNA1-4 (NCBI Acc. No.: KX665536, KX665537, KX665538, and MF476800) (Laufer *et al.*, 2018b) and the generated P-type infectious clone comprising RNA1-5 (see above). For sugar beet inoculation, each bacterial culture carrying a single plasmid with a cDNA of one of the viral RNAs was grown on selective agar media for two days. Before inoculation, a laboratory spoon was used to scrap from each plate a similar amount of bacterial culture (until the spoon is completely filled). The different cultures were then mixed manually in an empty petri dish. The mixture of these cultures, containing plasmids coding for the entire set of genomic RNA elements (A-type: RNA 1-4; P-type: RNA1-5), was used for inoculation of 7 days old sugar beet seedlings. For this purpose, an insulin needle (BD Micro-Fine™, 0.3 mm needle diameter) dipped into the culture was used to puncture the seedlings at three different positions along the hypocotyl. *B. macrocarpa* plants were inoculated by agroinfiltration with an OD_{600} of 1 into both cotyledons and one true leaf as described before (Liebe *et al.*, 2020). Two leaves of 14-day-old *N. benthamiana* seedlings were inoculated by means of agroinfiltration with an OD_{600} of 0.2 (Laufer *et al.*, 2018a). For infection experiments with *B. vulgaris*, a susceptible (KWS03), *Rz1*-resistant (Beta4430) and *Rz1*+*Rz2* resistant (Angelina) genotypes were used (KWS SAAT SE & Co. KGaA).

Virus quantification

A double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was applied to measure the viral load of BNYVV in infected lateral roots of *Beta* species and in leaf material from *N*. *benthamiana*. Antibodies specific for BNYVV CP (AS-0737) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany, Braunschweig). The root material (100-150 mg) was grinded in sample buffer (1:20, w/v) for 45 seconds at 5,000 rpm using the Precellys 24 tissue homogenizer (Bertin Instruments). The ELISA was conducted according to the manufacturer's instructions. Raw absorbance values measured at 405 nm were corrected by subtraction of blank and buffer control. Only samples with an absorbance value higher than the mean of the healthy control plus three times standard deviation were considered positive.

Detection of BNYVV RNAs in *B. vulgaris* roots

RNA was extracted from plant tissue using the NucleoSpin RNA Plant kit (Macherey-Nagel). Approximately, 200 mg of sugar beet root material was used for RNA extraction according to the manufacturer's instructions. The quantity and quality of the RNA was checked with a DS-11 Series Spectrophotometer (Denovix). For cDNA synthesis, 1000 ng RNA was reverse transcribed into cDNA using RevertAid H Minus reverse transcriptase and Oligo(dT)18 primer (Thermo Fisher Scientific). Viral RNA was then detected by PCR (Primer: Table S1) and separated by agarose gel electrophoresis. Signals were visualized using GelRed and photographed using a UV transilluminator (Intas Science Imaging Instruments GmbH).

Statistical analysis

Statistical analysis was performed with SigmaPlot14 (SigmaPlot 14.0, Systat Software Inc.). The data were first tested for normal distribution ($p \leq 0.05$) using Kolmogorov-Smirnov test followed by Brown-Forsythe test to check for equality of group variances ($p > 0.05$). The data were analyzed using Student's t-test. When equality of variances cannot be assumed, Welch's t-test was used. Graphic representations of the data were created using Excel 2013 (Microsoft Corp.). Only positive values (data from infected plants) were included in the graphical representation and statistical analysis. In each graph, the standard deviation (SD) and significance (not significant (n.s.) = $p > 0.05$; * = $0.01 \leq p < 0.05$; ** = $0.001 \leq p < 0.01$; *** = $p < 0,001$) are displayed. Significant differences between treatment levels of one factor were performed using one-way ANOVA. Data in tables are presented as mean values ±SD (standard deviation).

Results

Sequence comparison and proof of infectivity

In the first step, we compared all ORFs from the generated P-type clone with the annotated P-type genome as well as with reference isolates from the A-and B-type (Table 1). The CP sequence of our clone was identical with the annotated P type and displayed the second highest homology to the A-type from BNYVV, whereas the B-type had the lowest homology. Housekeeping genes located on RNA1 and RNA2 were most similar to the P-type followed by the A-and B-type. The lowest homology (93,53%) was found between J- and P-type RNA5 encoded p26.

Table 1. Percentage of amino acid identities between the ORFs of the P-type cDNA clone from this study and sequences from reference isolates (A-, B-, and P-type) deposited at the NCBI database.

	RNA1	RNA2						RNA3	RNA4	RNA5
BNYVV	237K	75K RT	21K CP	42K TGB-p1	13K TGB-p2	15K TGB-p3	14K Cys-R	25K p25	31K p31	26K p26
A-type	99.62%	96.67%	98.94%	99.76%	98.31%	98.48%	100%	96.33%	95.39%	93.53%*
B-type	98.72%	96.33%	96.81	99.48%	98.31%	96.97%	94.49%	95.43%	97.69%	n.a.
P-type	99.86%	99.71%	100%	100%	100%	99.24%	100%	100%	99.29%	100%

Percent identity (%) was determined using NCBI Protein BLAST. Abbreviations: n.a., not applicable; RT, read-through; CP, coat protein; TGB, triple gene block; Cys-R, cysteine-rich. *Percent identity of the Asian J-type RNA5.

The infectivity of the generated P-type clone was proven in one experimental host (*N. benthamiana*) and two *Beta* species (*B. macrocarpa* and *B. vulgaris*). Additionally, the A-type clone was inoculated as control. The P-type clone was able to infect all host plants and moved systemically resulting in leaf symptoms (Fig. 1). There were no differences in the ELISA absorption values compared to the A-type (Table 2). Clear differences between the two BNYVV types were observed in the symptom severity. In *N. benthamiana*, discoloration of the leaves induced by the P-type appeared to be more severe compared to the A-type (Fig. 1A). *B. macrocarpa* plants infected with the P-type displayed intensive dwarfism and a reduction in leaf size that was less pronounced in the A-type (Fig. 1B). In *B. vulgaris*, the P-type induced crinkled leaves in addition to severe vein yellowing (Fig. 1C). The crinkled leaves were not

observed when the A-type was inoculated. The presence of the viral RNAs in systemically infected tissue was confirmed by PCR in all host plants (Fig. S1).

Table 2. Infectivity of the BNYVV P-type clone in three different host plants (*N. benthamiana, B. macrocarpa and B. vulgaris*) after agroinfiltration.

Plant	BNYVV A-type		BNYVV P-type		Mock	
	Mean A_{405}[a]	*SD*[b]	Mean A_{405}[a]	*SD*[b]	Mean A_{405}[a]	*SD*[b]
N. benthamiana	0.60	0.05	0.57	0.02	0.00	0.01
B. macrocarpa	0.99	0.04	0.90	0.04	0.04	0.01
B. vulgaris	0.77	0.17	0.90	0.23	0.00	0.01

[a] Mean absorbance values (A_{405}) were determined by double antibody sandwich ELISA in lateral roots of *B. vulgaris* and in leaves of *N. benthamiana* and *B. macrocarpa* plants (*n*=6, infection rate 100%). [b] SD = standard deviation.

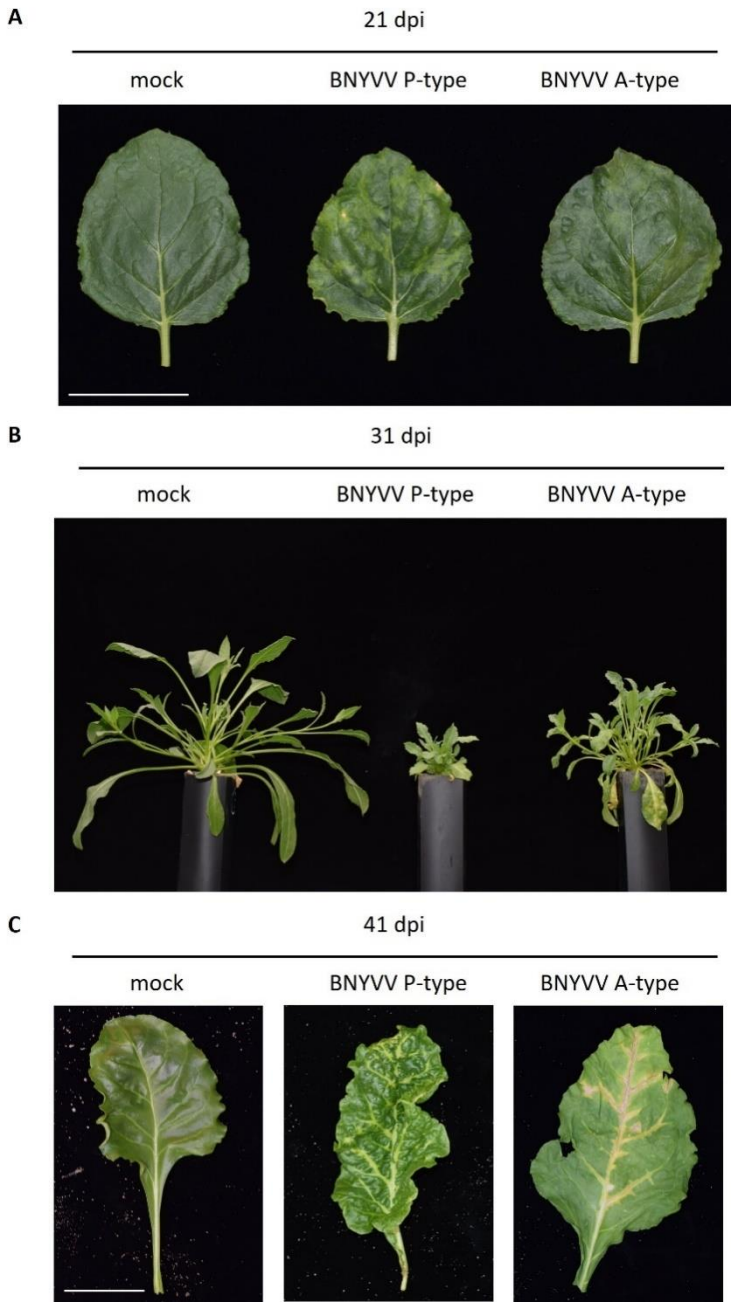

Figure 1. Phenotype of plants inoculated with BNYVV A-and P-type compared to non-inoculated plants (mock). **(A)** *N. benthamiana* leaves at 21 dpi; **(B)** whole plants of *B. macrocarpa* at 31 dpi; **(C)** *B. vulgaris* leaves at 41 dpi; Scale bar, 5 cm.

Effect of RNA5 on virus pathogenicity in a susceptible sugar beet variety

The next aim of this study was to determine the effect of the RNA5 from the P-type on virus accumulation and taproot weight in sugar beet. For this experiment, the A- and P-type cDNA clones were inoculated in a susceptible variety either with or without P-type RNA5. After 69 dpi, the P-type (with RNA5) induced the highest virus titer and the strongest reduction in taproot weight (Fig. 2A). This effect was lowered when RNA5 was not added to the inoculum.

The A-type displayed the lowest virus titer and reduction in taproot weight compared to the other inoculated variants. Inoculation of the A-type along with P-type RNA5 increased the virus titer and further reduced the taproot weight. However, there were no statistical differences in the virus titer and taproot weight between all inoculated variants. Plants inoculated with BNYVV A-type rarely showed foliar symptoms independent on the presence or absence of RNA5. The P-type also induced foliar symptoms with and without RNA5 (Fig. S2). A strong root beard was observed in all treatments and the phenotype did not differ between them, regardless of the virus type and presence of RNA5 (Fig. 2B). The presence or absence of RNA5 was confirmed in systemically infected leaves from all variants (Fig. 2C).

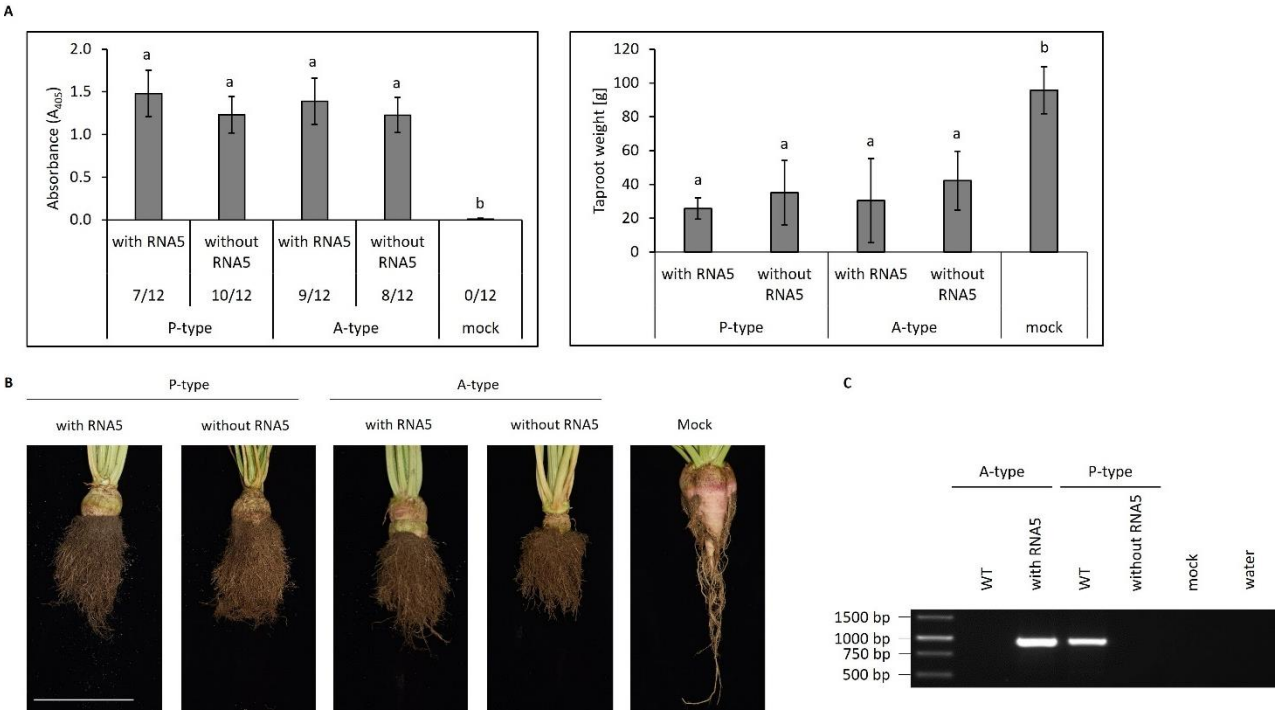

Figure 2. (A) Mean ELISA absorbance value (A_{405}) and taproot weight of BNYVV inoculated and non-inoculated (mock) sugar beet plants (69 dpi). Plants were either inoculated with the A- or P-type in the presence or absence of RNA5 (*n*=12). Vertical bars indicate standard deviation (SD) and significant differences are indicated as small letters ($p < 0.05$). Only infected samples were used for the mean calculation; the infection rate is indicated in brackets below each bar plot. **(B)** Root phenotype of all variants at 69 dpi. The scale bar represents 10 cm. **(C)** Confirmation of the presence or absence of RNA5 in all variants by RT-PCR detection of a fragment from RNA5 (886 bp) in lateral roots.

Effect of the P-type RNA5 on *Rz1* resistance-breaking

To analyze the resistance-breaking properties of the P-type, a susceptible and *Rz1* resistant variety was inoculated and the virus titer determined in lateral roots. The A-type clone was used as control because this clone was derived from a non-resistance-breaking population. The recombinant P-type could infect both varieties with a similar infection rate, although the virus titer was significantly reduced in the *Rz1* resistant variety (Fig. 3). In contrast, BNYVV could not be detected at all in *Rz1* resistant plants after inoculation with the non-resistance-breaking A-type clone (Fig. 3).

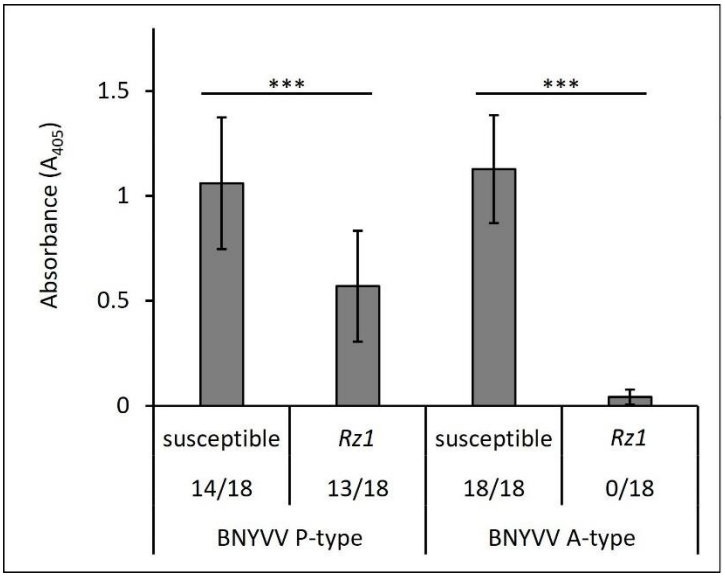

Figure 3. Mean ELISA absorbance value (A_{405}) determined in lateral roots of a susceptible and *Rz1* resistant variety (34 dpi). The plants (*n*=18) were either infected with the P- or A-type in two individual experiments (*n*=18). Vertical bars indicate standard deviation (SD) and horizontal bars indicate significant differences between treatments (*** = $p < 0{,}001$). Only infected samples were used for the mean calculation; the infection rate is indicated in brackets below each bar plot.

To elucidate the effect of RNA5 on resistance-breaking, we repeated this experiment and omitted the RNA5 cDNA from the inoculum. The absence of RNA5 had no effect on the infection rate or virus titer in the susceptible variety (Fig. 4). In contrast, both the infection rate and virus titer dropped in the *Rz1* resistant variety when RNA5 was not supplemented to the inoculum.

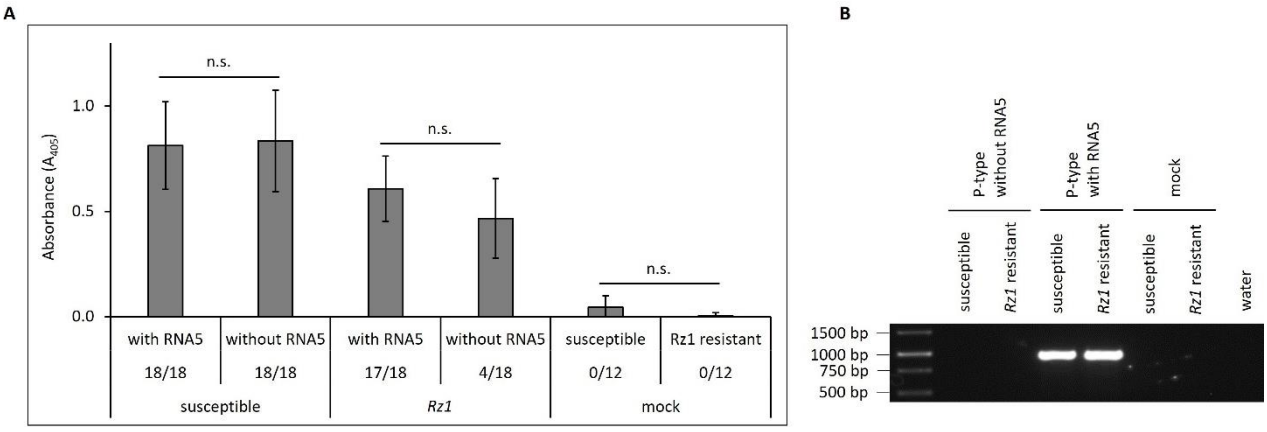

Figure 4. (A) Mean ELISA absorbance value (A_{405}) determined in lateral roots of BNYVV P-type and non-inoculated (mock) sugar beets. A susceptible and an *Rz1* resistant variety was mechanically inoculated with the P-type with and without RNA5 and lateral roots were harvested after 34 dpi (*n*=18). Vertical bars indicate standard deviation (SD), horizontal bars indicate which groups were compared ("n.s.": not significant). Only infected samples were used for the mean calculation; the infection rate is indicated in brackets below each bar plot. **(B)** Confirmation of the presence or absence of RNA5 in all variants by RT-PCR detection of a fragment from RNA5 (886 bp) in lateral roots.

Severe root symptoms could be observed in the *Rz1* variety when RNA5 was present, but plants seemed to be less stunted and the leaves were not crinkled when RNA5 was omitted (Fig. S3). Nevertheless, the P-type was still able to infect the *Rz1* variety to some extend despite the absence of RNA5. Finally, we tested the pathogenicity of the P-type clone in a double resistant variety carrying *Rz1* and *Rz2*. Here, the double resistant variety completely prevented an infection with the P-type (Fig. 5).

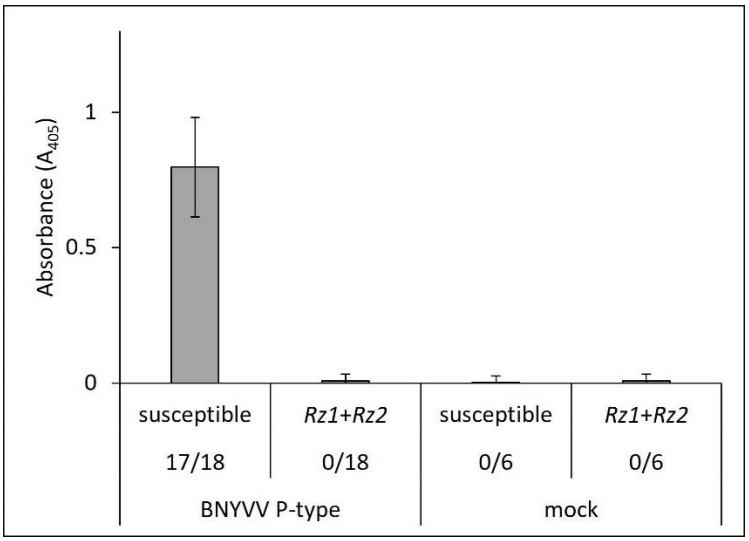

Figure 5. Mean ELISA absorbance value (A_{405}) determined in lateral roots of BNYVV P-type (RNA1-5) and non-inoculated (mock) sugar beets. A susceptible and a *Rz1+Rz2* resistant sugar beet variety was mechanically inoculated and lateral roots were harvested at 34 dpi (*n*=18). Vertical bars indicate standard deviation (SD). The infection rate is indicated in brackets below each bar plot.

Infectivity of genetic reassortments between A- and P-type

Finally, we aimed to prove whether the A- and P-type can form infective genetic reassortments due to their high sequence homology. For this purpose, we exchanged RNA1, RNA2 and RNA3 between both virus types and tested the infectivity in sugar beet. After 34 dpi, systemic symptoms could be observed in all variants confirming that the genetic reassortments were able to move systemically. The absorption values of the subsequent ELISA demonstrated that all reassortments could successfully infect the sugar beet plants and replicate in lateral roots (Table 3). In both virus types, the virus titers of the RNA1 and RNA2 reassortants did not differ from the virus titer measured in the wild type. Only in case of the RNA3 reassortants, we observed a significant drop in the virus titer for both virus types respectively (Table 3).

Table 3. Reassortment experiments between the A- and P-type clones. RNA1-3 of both types were exchanged with each RNA from the other type and mechanically inoculated into *B. vulgaris* seedlings by needle inoculation.

Reassortment		Mean A_{405} [a]	*SD*[b]	Infection rate
P-type background	WT	1.18^{A}	0.17	13/18
	A-type RNA1	1.01^{AB}	0.15	12/18
	A-type RNA2	1.22^{A}	0.25	12/18
	A-type RNA3	0.72^{B}	0.14	13/17
A-type background	WT	0.81^{ABC}	0.20	13/18
	P-type RNA1	0.76^{B}	0.15	16/18
	P-type RNA2	0.52^{CD}	0.07	15/18
	P-type RNA3	0.44^{D}	0.11	15/18
mock		0.01^{E}	0.01	0/12

[a]Mean absorbance values (A_{405}) were determined by double antibody sandwich ELISA in lateral roots of *B. vulgaris* plants. Letters behind the mean A_{405} values indicate significant differences between the varieties (n=12, one-way ANOVA ($p < 0.05$). The reassortants with the A-type background and the P-type background were analyzed separately. [b]SD = standard deviation.

Discussion

A reverse genetic system is a valuable tool to study the biology and pathogenicity of BNYVV virus types as it allows the removal and exchange of RNA components, and the infection is not influenced by abiotic or biotic side-effects when natural populations are used. In this study, we generated the first infectious cDNA clone of the BNYVV P-type with five RNA components derived from a virus population collected in Pithiviers. We confirmed the infectivity of the clone in the two experimental hosts *N*. *benthamiana* and *B*. *macrocarpa*, and, moreover, the P-type clone reassembled rhizomania-like symptoms in the crop plant sugar beet. Thus, it is reasonably assumed that the genome integrity was not affected by the artificial inoculation at least in case RNA1-3 which are absolutely necessary for symptom development and movement in sugar beet (Lauber *et al.*, 1998; Tamada *et al.*, 1999). Furthermore, the formation of the ncRNA3 must have occurred as it is required for systemic movement (Flobinus *et al.*, 2018). The occurrence of deletion mutants in case of RNA4 and RNA5 cannot

be excluded as these two RNAs are principally not necessary for an infection here. We think the strong effect of RNA5 in the *Rz1* resistant genotype suggests that the P26 ORF located on this RNA remained intact. Similarly, a complete loss of RNA4 after inoculation is very unlikely as our A-type clone retained vector transmissibility after agroinfection in *B*. *macrocarpa* (Laufer *et al.*, 2018b). Nevertheless, we should keep in mind that defective RNAs might occur, when artificial inoculation in combination with a cDNA clone is used for infection.

In all plant species, we could observe somewhat stronger systemic symptoms induced by the P-type, but there were no differences in the virus titer compared to the A-type clone. Therefore, these observations should be seen with caution as development of systemic leaf symptoms can be very variable and may be linked also to our infection method based on *R. radiobacter.* Regarding the taproot, both virus types heavily reduced the taproot weight, induced massive lateral root development and accumulated a high virus titer in a susceptible sugar beet variety. The P-type with RNA5 tend to have the strongest effect, but there were no statistical differences between the variants. Therefore, our results demonstrate that RNA5 is dispensable for the P-type to infect and to induce taproot symptoms in a susceptible variety. This is in accordance with a previous study from Iran reporting the presence of pathogenic P-type isolates lacking RNA5 in sugar beet (Mehrvar *et al.*, 2009). To sum up, we found similar pathogenic properties between the A- and P-type after infection of a susceptible sugar beet variety. An effect of the RNA5 on symptom severity as reported for the Asian J-type was not observed (Tamada *et al.*, 2020). This could be either explained by distinct pathogenic properties of J- and P-type RNA5 or experimental differences due to the usage of natural virus populations by the above-mentioned study.

In terms of resistance-breaking, we could clearly show that the recombinant P-type is able to overcome *Rz1* resistance as previously reported for natural populations of this virus type (Pferdmenges *et al.*, 2008; Bornemann & Varrelmann, 2011; Bornemann *et al.*, 2015). In contrast, the A-type cDNA clone derived from a non-resistance-breaking population failed to infect the *Rz1* resistant variety at all. However, the P-type accumulated to a significantly lower virus titer in the *Rz1* variety indicating that the aggressiveness of the P-type is still reduced compared to the susceptible variety. Interestingly, this reduction of the virus titer could also be observed in resistance tests using natural P-type populations from Pithiviers (Heijbroek *et al.*, 1999; Bornemann & Varrelmann, 2011; Bornemann *et al.*, 2015). Furthermore, our reverse

genetic system allowed us to demonstrate that the efficiency to overcome *Rz1* is associated with the presence of RNA5. The infection rate dropped remarkably from 94% with RNA5 to only 22% without RNA5 and the virus titer was also reduced. Therefore, we can conclude that P- and J-type RNA5s share similar properties regarding *Rz1* resistance-breaking (Tamada *et al.*, 2020). Nevertheless, the P-type without RNA5 could infect a few (4/18) *Rz1* resistant plants that was not the case for the A-type clone. We have shown with our A-type clone that specific tetrad variants in p25 (AYPR, VCHG, VLHG) mediate *Rz1* resistance-breaking leading to a similar virus titer in susceptible and resistant varieties (Liebe *et al.*, 2020). The p25 sequence from the P-type carries the tetrad variant SYHG which is unique to this virus type and absent in A- or B-type populations (Schirmer *et al.*, 2005; Chiba *et al.*, 2011). Furthermore, natural P-type populations display no variability in the tetrad of p25 as observed for A-type populations. Our data indicate that the tetrad SYHG allows virus accumulation, but with reduced infection efficiency in *Rz1* resistant plants. Consequently, this suggests that the RNA5 is the main driver of *Rz1* resistance-breaking in P-type populations. Furthermore, a recent study has shown that the *Rz1* resistance breaking properties of P-type RNA5 can be transferred to the A-type when the cDNA clone is supplemented with P-type RNA5 (Liebe *et al.*, 2020). This confirms a second resistance mechanism, independent of the p25 tetrad. However, a double resistant variety with *Rz1*+*Rz2* prevented infection with the P-type which means that the resistant-breaking effect of RNA5 is specific to *Rz1*.

Finally, we investigated for the first time the ability of the A- and P-type to form viable reassortments in sugar beet. Despite their sequence divergence, the RNAs 1-3 could be exchanged between both virus types without affecting the ability of the virus to accumulate in lateral roots and to move systemically. This has been also observed for the more distantly related *Beet soil-borne mosaic virus* with the BNYVV A-type and further confirms that the function of the BNYVV RNAs highly conserved within the sugar beet infecting *Benyviruses* (Ratti *et al.*, 2009; Laufer *et al.*, 2018a). Compared to the wild type viruses, only the exchange of RNA3 reduced the virus titer significantly in our study. The p25 ORF on RNA3 displays the lowest sequence homology between the A- and P-type suggesting an adaption to the virus type as also indicated by previous phylogenetic studies (Chiba *et al.*, 2011). This may explain the lower virus titer when the RNA3 was replaced in the A- and P-type. Principally, our results show that a formation of new viral variants by exchange of RNA components is possible.

Interestingly, both virus types can occur in mixed infection which is a prerequisite for such evolutionary events. However, we think this is very unlikely as co-infection experiments with our BNYVV A-type clone imply that super-infection exclusion will occur when such closely related virus types infect the same cell (Laufer *et al.*, 2018a).

Our study highlights the close evolutionary relationship between the A- and P-type, but we could also demonstrate distinct pathogenicity properties in *Rz1* resistant varieties. The genetic composition as well as high sequence similarities between the different BNYVV virus types led to the assumptions that all virus types arose from one ancestor population (Chiba *et al.*, 2011). This population is assumed to be originated in East Asia and spread from there worldwide along with the sugar beet cultivation which provoked the development of distinct virus types. It is speculated that the P-type was introduced to Pithiviers originally as an Asian A-type isolate (with J-type RNA5) present in soil adhering to mulberry tree plantlets imported for multiplication and used for feeding silkworms (Meulemans *et al.*, 2003). The Pithiviers region has a long history of sugar beet breeding for resistance which may have forced the evolution of the P-type after the introduction (Biancardi *et al.*, 2002; Galein *et al.*, 2018). Until now, the P-type displays only a minor distribution despite its clear fitness advantage in *Rz1* resistant varieties that are grown in all European sugar beet growing areas. However, a recent analysis of many BNYVV populations from Japan revealed that the incidence of Asian A-type isolates possessing a RNA5 (J-type) increased in the past decades (1991-2019) likely to the introduction of resistant varieties (Nakagami *et al.*, 2021). Such current population studies are missing in Europe, but our results strengthen their importance.

Author statements

Authors and contributors

Maximilian M Muellender (https://orcid.org/0000-0001-5278-3674): Formal analysis; Investigation; Methodology; Project administration; Resources; Validation; Visualization; Writing – original draft

Mark Varrelmann (https://orcid.org/0000-0003-1014-5185): Conceptualization; Project administration; Supervision; Writing – review & editing

Edgar Maiss: Conceptualization; Investigation; Project administration; Supervision; Writing – review & editing

Sebastian Liebe (https://orcid.org/0000-0002-4728-9548): Conceptualization; Formal analysis; Investigation; Methodology; Project administration; Supervision; Writing – review & editing

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Yilmaz, N.D.K., Arli-Sokmen, M. & Kaya, R. (2018) p25 pathogenicity factor deletion mutants of beet necrotic yellow vein virus occurring in sugar beet fields in Turkey. *Journal of Plant Diseases and Protection*, 125(1), 89–98. Available from: https://doi.org/10.1007/s41348-017-0142-4.

Supporting Information

Figures

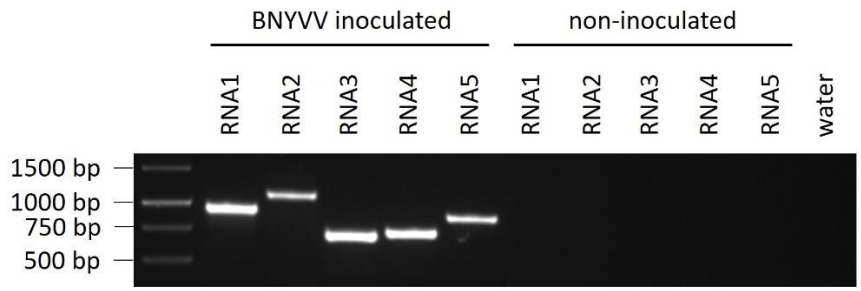

Figure S1. PCR detection of fragments from all five P-type RNAs (RNA1 – 816 bp, RNA2 – 1021bp, RNA3 – 678 bp, RNA4 – 693 bp, RNA5 - 886 bp) in lateral roots of *B. vulgaris* plants using agarose gel electrophoresis. Additionally, a water control is shown to prove the purity of the RT-PCR reaction. Samples were taken at 41 dpi.

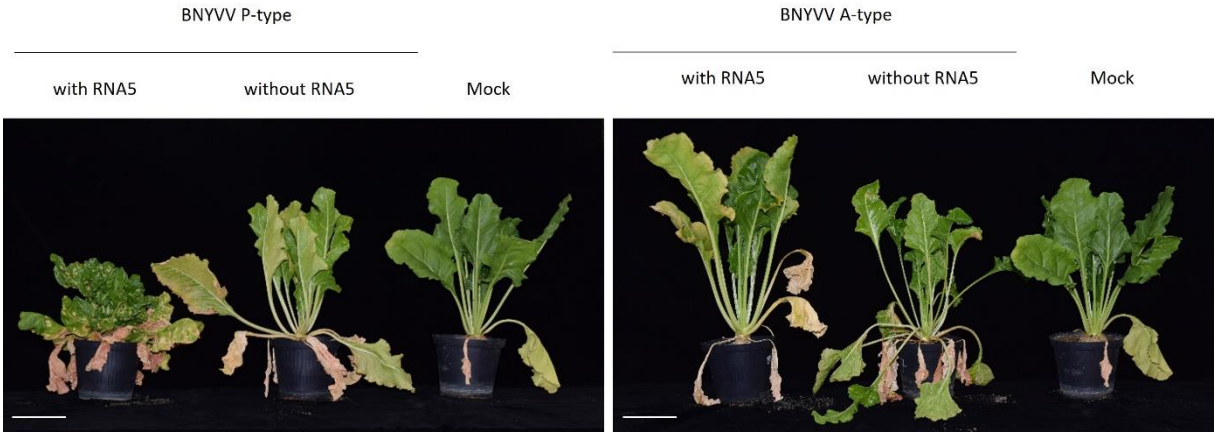

Figure S2. Phenotypes of susceptible sugar beet plants inoculated with the BNYVV A- or P-type clone in the presence or absence of RNA5 compared to non-inoculated plants (mock). Pictures were taken at 35 dpi. Scale bar: 10 cm.

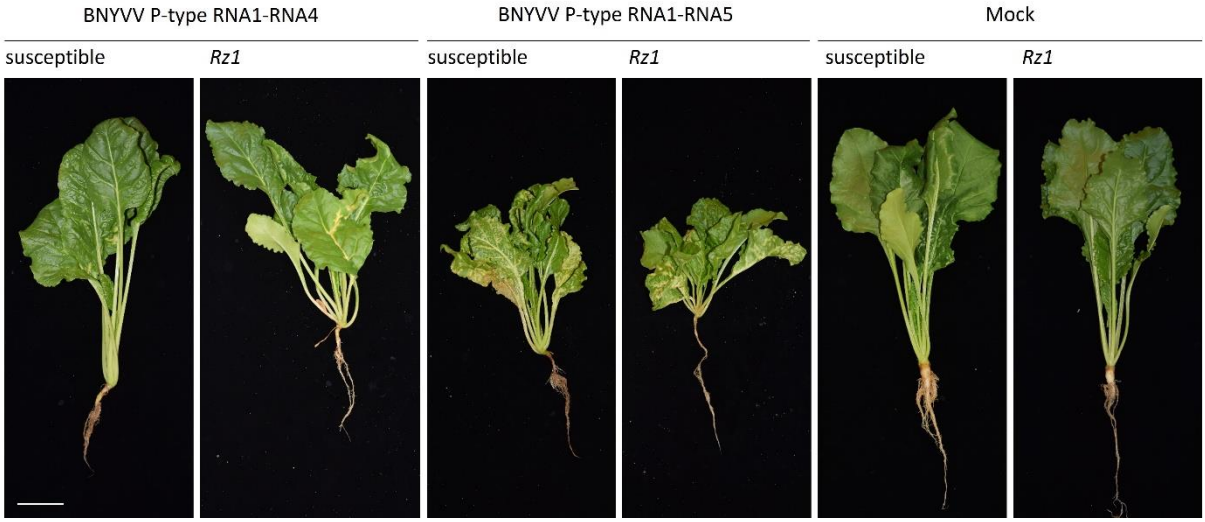

Figure S3. Phenotypes of susceptible and *Rz1* resistant sugar beet plants inoculated with BNYVV P-type with and without RNA5 compared to non-inoculated plants (mock). Pictures were taken at 35 dpi. Scale bar: 5 cm.

Tables

Table S1. List of all primers used in this study for vector and RNA amplification.

Primers used for amplification of pDIVA:

Papa_sf	CCTCTCCAAATGAAATGAACTTCCTTATATAG
Mama_sf	GGGTCGGCATGGCATCTCCACCTCCTC

Primers used for cDNA synthesis of BNYVV P1-P5:

RACE-BOE1	GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTT(AGC)
CPEC_dT22	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTT
BNYVV_CEP_1.3	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTTTATATCAATATAC
BNYVV_CEP_2.3	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTTCAATATACTG
BNYVV_CPE_2.5	AGGAAGTTCATTTCATTTGGAGAGGAAATTCTAACTATTATCTCC
NEW_BNYVV_2.2	GAGAAAACACTAGTAGAGGATGGGTATAAATG
BNYVV_2752AsM	GAGATGCCATGCCGACCCCATTTATACCCATCCTCTACTAGTG
BNYVV_3.1s	AGGAAGTTCATTTCATTTGGAGAGGAAATTCAAAATTTACCATTACATATTG
BN_P34as	GAGATGCCATGCCGACCCGTCATATACTGACAAAG
BN_P5as	GAGATGCCATGCCGACCCGTCAATACACTGACAGA

Primers used for PCR of BNYVV P1-P5:

RNA_1:	
BNYVV_1.1As	TGCGCACCGGTCGCCTCGAACAAAT
BNYVV_CPE_1.5	AGGAAGTTCATTTCATTTGGAGAGGAAATTCGATTCTTCCCATTC
BNYVV_1.SP1	GTTCGAGGCGACCGGTGCGCAGACT
BNYVV_1.2AsM	AGGAAGTTCATTTCATTTGGAGAGGAATGTCGACGATGATTTTGTCAATATG
BNYVV_1.SP2	AATGTCGACGATGATTTTGTCAATATG
BNYVV_1.3sP	GAGATGCCATGCCGACCCCATATTGACAAAATCATCGTCGACATT
BNY_1.1AsM	GAGATGCCATGCCGACCCTGCGCACCGGTCGCCTCGAACAAAT
BNY_1.2sP	AGGAAGTTCATTTCATTTGGAGAGGGTTCGAGGCGACCGGTGCGCAGACT

BNYVV_1.2As	CATATTGACAAAATCATCGTCGACATTC
RNA_2:	
BNYVV_CPE_2.5	AGGAAGTTCATTTCATTTGGAGAGGAAATTCTAACTATTATCTCC
BNYVV_2752AsM	GAGATGCCATGCCGACCCCATTTATACCCATCCTCTACTAGTG
BNYVV_CEP_2.3	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTTTTCAATATACTG
NEW_BNYVV_2.2	GAGAAAACACTAGTAGAGGATGGGTATAAATG
NEW_BNYVV_2.1	CATTTATACCCATCCTCTACTAGTGTTTTCTC
RNA_3:	
CPEC_dT22	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTT
BNYVV_3.1s	AGGAAGTTCATTTCATTTGGAGAGGAAATTCAAAATTTACCATTACATATTG
polyAMAMAs	AAAAAAAAAAAAAAAAAAAAAAGGGTCGGCATGGCATCTCCAC
BN_P3as	GAGATGCCATGCCGACCCGTCATATACTGACAAAG
BNYVV_P3_4A	GTCATATACTGACAAAGAACCCTA
RNA_4:	
BN_P4s	GAAGTTCATTTCATTTGGAGAGGAAATCAAATCTCAAAATATATATTTG
BN_P4as	GAGATGCCATGCCGACCCGTCATATACTGACAGAG
polyAMAMAs	AAAAAAAAAAAAAAAAAAAAAAGGGTCGGCATGGCATCTCCAC
BNYVV_P34A	GTCATATACTGACAAAGAACCCTA
RNA_5:	
BN_RNA5Ps	AGGAAGTTCATTTCATTTGGAGAGGAAATTCAAAGTACTTTCATATTG
BN_P5as	GAGATGCCATGCCGACCCGTCAATACACTGACAGA
polyAMAMAs	AAAAAAAAAAAAAAAAAAAAAAGGGTCGGCATGGCATCTCCAC
BNYVV_P5A	GTCAATACACTGACAGAGAACCCTA

Primers used for partial PCR detection of BNYVV RNA1-5:

RNA_1:	
#366_RNA1_fw	CAGTGGGGCTTTGTACAC
#367_RNA1_rv	CATGAGTTCTCGCTCACC
RNA_2:	
RNA2-CP-fw	GCCCTACTTTAAATATAGGTGCG
RNA2-CP-rv	AGGATATAATAGTGCCCGCTTC
RNA_3:	
#3_ P25_F	ATGGGTGATATATTAGGCG
#4_ P25_R	CTAATCATCATCATCAACAC
RNA_4:	
RNA4-P31-fw	CTGGGATCCAGTCTATCAGTAAG
RNA4-P31-rv	CACATAAACCTTACCATAGCAAGG
RNA_5:	
RNA5-P26-fw	GTTTTTCCGCTCGCACAAGCG
RNA5-P26-rv	CGAGCCCGTAAACACCGCATA

4. General discussion

4.1 Interaction of p25 with the auxin signaling pathway

Auxins are plant hormones involved in many different metabolic processes such as vascular tissue formation, tropistic responses, apical dominance, flower and fruit development but also in cellular processes, such as cell division, enlargement and differentiation (reviewed in Davies, 1995; Reed, 2001; Ori, 2019). The control of such developmental processes is regulated by finely tuned transcriptional mechanisms, such as the auxin signaling pathway. Since the development of the LRs is also controlled by auxin (reviewed in Lavenus *et al.*, 2013) and the most characteristic symptom of a BNYVV infection is the massive proliferation of LRs, it seems reasonable to assume that BNYVV can somehow interfere with the auxin signaling pathway. In fact, BvIAA28 has already been found to interact with the viral pathogenicity factor p25 in a previous study (Thiel & Varrelmann, 2009). Since it was not clear yet, if p25 also interacts with other members of the Aux/IAA family from *B. vulgaris*, the next step was to test the other 12 Aux/IAA proteins from sugar beet for interaction with p25. Yeast two-hybrid (Y2H) was used to identify possible interactions and bimolecular fluorescence complementation (BiFC) as well as co-immunoprecipitation (co-IP) were used to confirm these findings.

After identifying six additional sugar beet Aux/IAA candidates to interact with p25 by Y2H (BvIAA2, BvIAA6, BvIAA9, BvIAA13, BvIAA14 and BvIAA33), it was possible to confirm only two of these interactions by BiFC and co-IP, namely BvIAA2 and BvIAA6. All these assays, Y2H, BiFC and Co-IP are prone to false positive or false negative results. An interaction detected in yeast by Y2H does not necessarily occur *in planta*. Such differences are well known, and therefore further *in planta* tests must always be performed to confirm such plant/virus interactions (MacFarlane & Uhrig, 2008). To produce valid and meaningful results with protein-protein interaction assays, it is important to use all necessary controls as well as to check interactions in at least two independent assays. The transfer from yeast to plant, for example, is quite difficult because plant proteins which are important for the interaction or correct folding of the proteins could be absent in yeast cells (Niemiro *et al.*, 2020). Another essential factor in which the organisms differ are posttranslational modifications which are crucial for possible interactions (Garcia *et al.*, 2007). For example, phosphorylation and *O*-GlcNAcylation of the

CP from plum pox virus (PPV) were found to be crucial for plant-virus interactions (Martínez-Turiño *et al.*, 2018; Hervás *et al.*, 2020). Therefore, to reduce host specific differences, one yeast (Y2H) and two plant (BiFC + co-IP) bioassays were used to identify interactions of Aux/IAA proteins from sugar beet with p25 from BNYVV as exemplified before with BvIAA28 and p25 (Thiel & Varrelmann, 2009). The fact, that the interaction of BvIAA2 and BvIAA6 with p25 can only be detected with degradation stable protein Aux/IAA variants in the co-IP assays clarifies how prone such tests are to false results. As mentioned above, Aux/IAA proteins are very short-lived proteins as long as no amino acid alterations are introduced to prevent protein degradation (reviewed in Reed, 2001). Without the degradation stable variants, it seemed, that the interaction cannot be confirmed by co-IP but these false negative results were due to the short-lived nature of the Aux/IAA proteins. During the methodical procedure, the plant cells in which the interacting proteins were produced are disrupted by mechanic and chemical lysis. This must be done under native conditions so as not to affect the interaction. Presumably, the Aux/IAA proteins are degraded in the proteasome during this step and thus can no longer be detected as interaction partners. The degradation-stable variants of the Aux/IAA proteins prevented this, so that the Aux/IAA proteins could still be detected.

In addition, it must be mentioned that just because interactions cannot be detected in these assays does not mean that the interaction does not occur in the host organism. This might be due to the biology of plant-virus interactions. The virus relies on the plant having pro-viral host factors, *e.g.* plant proteins that are important for viral replication and distribution (reviewed in Yadav & Chhibbar, 2018; Garcia-Ruiz, 2019). For example, if a plant lacks the translation initiation factor eIF(iso)4E, this can abolish susceptibility to potyviruses (Lellis *et al.*, 2002) or it has been found that pectin methylesterase is required for cell-to-cell movement of TMV (Chen & Citovsky, 2003). Some interactions may involve additional viral or host factors that are only encoded in the natural hosts. It is possible that p25 requires additional host proteins to interact with certain Aux/IAA proteins. Such an interaction of three proteins has been shown for the movement protein (MP) of cucumber mosaic virus with the viral 1a and 2a proteins (Hwang *et al.*, 2005). Indeed, BNYVV p25 appears not only to interact directly with Aux/IAA proteins. Recent studies also showed differentially regulated micro RNAs (miRNAs) involved in auxin signaling upon BNYVV infection at least in *N. benthamiana* and *B. macrocarpa* (Liu *et al.*, 2017; Liu *et al.*, 2020). Another aspect might be the dynamic of a

virus infection on molecular level. Gil and coworkers demonstrated in 2018, that BNYVV pathogenesis is a highly dynamic process. They showed that mRNA expression levels of some *EXPs* and *LBD* TFs are differentially regulated in LRs of BYNVV infected *B. vulgaris* plants between four and six weeks post infection (Gil *et al.*, 2018). Both genes are auxin responsive genes which might be also regulated by BvIAA2, BvIAA6 or BvIAA28. Assuming that BNYVV is responsible for the regulation of these genes during pathogenesis by interaction with the auxin signaling pathway, this illustrates a dynamic process and maybe a dynamic interaction of proteins. What exactly causes these dynamics is not known but it could be also explained by additional factors. Perhaps the interaction is influenced by the developmental stage of the plant, for example, by proteins that are formed only in early developmental stages. However, whether and how exactly such additional factors are involved in the interaction, remains unclear, but it should be emphasized that interaction studies often only give a limited view about a highly dynamic interaction process.

4.2 The interaction of p25 with Aux/IAA proteins requires the full-length, sequence identical proteins

To further characterize the interaction, the subcellular localization of BvIAA2 and BvIAA6 with and without p25 presence was determined. Like BvIAA28 (Gil *et al.*, 2018), the subcellular localization of BvIAA2 and BvIAA6 revealed that they exclusively accumulate in the nucleus when expressed individually. This is certainly due to the fact that all three Aux/IAA proteins encode two NLS signals similar to those of other Aux/IAA proteins (Abel *et al.*, 1994; Wu *et al.*, 2012). It has been shown that both NLS signals are responsible for nuclear localization and that absence and mutation of one of the NLS regions caused Aux/IAA proteins to be detected not only in the nucleus, but also in cytoplasm probably due to protein diffusion (Ludwig *et al.*, 2014; Wu *et al.*, 2017).

All Aux/IAA proteins that interact with p25 encode both NLS signals and exclusively accumulate in the nucleus (Gil *et al.*, 2018). The pathogenicity factor p25 localizes both in the nucleus and in the cytoplasm, most probably due to an NES signal in addition to an NLS signal in this protein (Vetter *et al.*, 2004). The localization of p25 did not change, when co-expressed with BvAA2 and BvIAA6. This is in common with other observations made with BvIAA28, where no altered localization of p25 was observed as well (Gil *et al.*, 2018). In contrast, the

localization of BvIAA28 was found to change towards the cytoplasm when co-expressed with p25 (Gil *et al.*, 2018). This alteration of the localization had been proposed to inactivate the function of BvIAA28 as a transcriptional regulator. Therefore, it is also interesting to investigate this for BvIAA2 and BvIAA6. Unlike BvIAA28, the subcellular localization of BvIAA2 and BvIAA6 was not altered by p25. Neither the BiFC assay nor co-expression of both labeled proteins revealed a change in subcellular localization into the cytoplasm, the cellular compartment of the 26S proteasome. On the one side, it might be possible that the Aux/IAA proteins are immediately degraded due to their short-lived biology (Abel *et al.*, 1994). This degradation might be too fast to visualize the re-localization microscopically, but consequently it should then as well not be possible to detect the altered subcellular localization of BvIAA28 (Gil *et al.*, 2018). On the other site, it is possible, that the interaction of p25 with BvIAA2 and BvIAA6 differs from the interaction with BvIAA28. It might be possible, that the interaction leads to a stabilization of the Aux/IAA proteins and protects them from degradation. This would allow the proteins to continue interacting with ARFs and act as transcription factors independent of the auxin level within the cell. This is also supported by the fact that the mRNA levels of *BvIAA2*, *BvIAA6* and *BvIAA28* were not significantly altered at different stages of BNYVV infection assayed via RT-qPCR (28, 44 and 66 dpi). Often Aux/IAA proteins regulate their own translation, which would lead to an increase in mRNA levels if the proteins are degraded. Such a stabilization of an Aux/IAA protein with the interacting ARF has been described for the viral protein P2 from RDV (Jin *et al.*, 2016). Another possibility is that the interaction of p25 with BvIAA2 and BvIAA6 results in inhibition of the interaction of the Aux/IAA proteins with ARFs, but without re-localization into the cytoplasm. First, it would explain the RT-qPCR results, and second, it would be consistent with the hypothesis that p25 inhibits the activity of BvIAA2 and BvIAA6 as transcriptional regulators. Based on the results, it can be concluded that there is no re-localization into the cytoplasm of BvIAA2 and BvIAA6 by p25. Whether their function as transcriptional regulators is nevertheless interrupted or not, could not be clarified.

Furthermore, it was assumed that p25 interacts with specific domains of the Aux/IAA proteins to disrupt the interaction of Aux/IAA proteins with ARFs. In case of BvIAA28, p25 has been shown to interact with DI and DII (Gil *et al.*, 2018). To identify interacting regions of both proteins, domain mapping of BvIAA2 and BvIAA6 as well as random mutagenesis of p25 was

performed. It was found that the interaction of both Aux/IAA proteins with p25 is extremely specific. Any changes in the proteins, such as deletions of the Aux/IAA domains or insertions of five amino acids randomly into the p25 protein, led to a complete loss of interaction. Additionally, the NLS as well as the NES signal was knocked out via amino acid exchanges. Via the knockout of the NLS, it would be possible to prevent the import of p25 into the nucleus and thus spatially interrupt the interaction. Interaction with p25 in the cell nucleus could still be possible with a deleted NES, but p25 would no longer be able to perform the shuttling function and transport the Aux/IAA proteins out of the cell nucleus. Incorporated into the infectious cDNA clone, these mutants would provide new insights into whether the shuttling function of p25 is at all important for infection and for BNYVV induced symptoms.

However, for such experiments, it must be ensured that the interaction of p25 and the Aux/IAA proteins is not affected. Yet even these single amino acid substitutions led to a complete loss of interaction with BvIAA2 and BvIAA6, respectively. As protein synthesis was detected by western blot, it must be assumed that the sequence identical, unchanged wild type (wt) proteins are required for the interaction. It can therefore be assumed that this interaction is extremely specific and interrupted by the smallest alterations of the interacting partners. In fact, such a specify has been also shown for p26, the symptom enhancer encoded on the P- and J-type. The ability to induce necrosis of local lesions in *C. quinoa* relies on the wt p26 protein, indicating that the entire protein is required, rather than a specific domain (Link *et al.*, 2005). Even with p25, such specificity has already been shown previously. Sequence variations in the p25 protein disrupt its ability to self-interact (Klein *et al.*, 2007). Together with these results, it can be confirmed that p25 is a highly conserved protein whose activity is based on the full-length, sequence identical protein and almost any artificial changes lead to loss of function. Most importantly, the results of this study show that the interaction itself is highly specific and equally susceptible to changes of one of the interaction partners. The fact that similar approaches to identify potential interacting domains in BvIAA2 and BvIAA6 were successful for the interaction of p25 with BvIAA28 (Gil *et al.*, 2018), indicates differences in the structure of these Aux/IAA proteins. Such structural variation of Aux/IAA proteins can be caused by intrinsically disordered regions (IDRs). IDRs are polypeptide segments that contain a high proportion of polar or charged amino acids, mediating a variation in three-dimensional conformations of the protein (Uversky *et al.*, 2000; reviewed in Babu, 2016). The possibility of

different conformations allows proteins with IDRs to interact with large number of partners specifically (Rogers *et al.*, 2014; reviewed in Babu *et al.*, 2012; Flock *et al.*, 2014). Depending on the interacting proteins, IDRs allow changing the three-dimensional conformation of the protein (Niemeyer *et al.*, 2020). Thus, IDRs affect interactions that regulate stress responses, development, metabolic and signaling pathways (reviewed in Covarrubias *et al.*, 2020). In the case of Aux/IAA proteins, IDRs has been described before and proposed to provide structural flexibility for interaction and proper positioning on *e.g*. Cullin RING-type E3 ubiquitin ligases TIR1 (Niemeyer *et al.*, 2020). This makes IDRs prone to conformational changes due to interaction with other proteins. However, these regions are also very sensitive to changes of amino acid sequence when interacting with primarily ordered regions of other proteins (reviewed in Mishra *et al.*, 2020) and *in silico* analysis predicts that p25 is an entirely ordered protein (data not shown). To sum up these results, it has been shown that the interaction of p25 with BvIAA2 and BvIAA6 is extremely conserved. This indicates a high degree of specialization of the interaction between host and virus proteins. It was shown that the complete structure of both partners is crucial for the interaction.

4.3 Orthologues of the interacting Aux/IAA proteins are involved in LR formation in *A. thaliana*

A multiple sequence alignment of all Aux/IAA proteins from sugar beet with the Aux/IAA proteins of the well-studied model plant *A. thaliana* was performed, to find orthologous proteins. Based on this, the Aux/IAA proteins can be investigated structurally and functionally to draw first conclusions about the interaction partners of p25 and to investigate the Aux/IAA family of *B. vulgaris* structurally. First, it can be stated that the four conserved functional domains could be identified in almost all Aux/IAA proteins from sugar beet. This indicates that most of these proteins are canonical Aux/IAA proteins, except for BvIAA4.2 and BvIAA33. These two proteins show only very weak, irregular homologies in domain II. Moreover, the invariant base doublet 'KR' between domain I and II cannot be found at all, a nuclear localization signal that acts together with the basic amino acids in domain II (Wu *et al.*, 2012). These special Aux/IAA proteins do not share the typical four-part structure of canonical Aux/IAA proteins, so they are classified as non-canonical. Since non-canonical Aux/IAA proteins can be found in many other plant species such as rice (Jain *et al.*, 2006), maize (Wang *et al.*, 2010), wheat (Qiao *et al.*, 2015) or cotton (Su *et al.*, 2022), it was not surprising to

identify this type of Aux/IAA proteins in sugar beet as well. Nevertheless, this is the first time these proteins are described in *B. vulgaris*.

Based on the multiple sequence alignment of the Aux/IAA proteins from *A. thaliana*, it was possible identify protein clusters containing proteins involved in LR development and root hair formation in a maximum likelihood tree (reviewed in Reed, 2001; Luo *et al.*, 2018). The model plant *A. thaliana* is genetically much more accessible, allowing knocking out (loss-of-function) or stabilization (gain-of-function) (Audran-Delalande *et al.*, 2012) of Aux/IAA proteins to study their function. Interestingly, the interacting Aux/IAA proteins BvIAA2, BvIAA6, and BvIAA28 were grouped in these clusters, showing a high sequence homology. The *Arabidopsis* proteins AtIAA18 and AtIAA28, which cluster together with BvIAA2, and AtIAA1 that clusters together with BvIAA6, are negative regulators (transcriptional repressors) of LR formation and their auxin-mediated degradation is required for proper LR development. Expression of degradation stable, gain-of-function variants of these proteins reduced LR development even in the presence of exogenously supplemented auxin (Rogg *et al.*, 2001; Fukaki *et al.*, 2002; Uehara *et al.*, 2008; Notaguchi *et al.*, 2012). Such negative regulators are also found among *Arabidopsis* proteins that cluster together with BvIAA28, namely AtIAA14/SLR and AtIAA16 (Fukaki *et al.*, 2002; Rinaldi *et al.*, 2012). However, expression of gain-of-function variants of two other Aux/IAA proteins (AtIAA7/AXR2; AtIAA17/AXR3) from this cluster led to an increased number of LRs, indicating an enhanced auxin response (transcriptional activators) (Leyser *et al.*, 1996; Nagpal *et al.*, 2000). In summary, although the sequence homology does not allow us to draw definite conclusions about the function of all three interacting Aux/IAA proteins or whether they are transcriptional repressors or activators, it has been shown that orthologs from *A. thaliana* are unambiguously assigned to root formation. This is the first evidence that the identified Aux/IAA proteins interacting with p25, BvIAA2, BvIAA6, and BvIAA28 are involved in LR formation.

4.4 *BvIAA2*, *BvIAA6* and *BvIAA28* are involved in root development

One method to study the biological function of different Aux/IAA proteins is the deletion of the gene of interest or the insertion of loss-of-function mutations. For example,

loss-of-function mutants of *ARF* genes from *A. thaliana* clarified the role of ARF7 and ARF19 in LR formation (Overvoorde *et al.*, 2005). In case of sugar beet, it is however quite difficult to inhibit protein synthesis or to silence its expression because the generation of transgenic *B. vulgaris* plants is rather difficult and inefficient. Virus-induced gene silencing (VIGS) is one way to silence a gene of interest and thus to inhibit protein synthesis without a transgenic approach which had been already used in different plant species (Liu *et al.*, 2002; Gao *et al.*, 2011; Hayward *et al.*, 2011; reviewed in Bekele *et al.*, 2019). Moreover, this method was even used for functional analysis of different Aux/IAA proteins (Feng *et al.*, 2020; Su *et al.*, 2022). In the case of sugar beet, this method has shown to be possible, but with a very low efficacy and poor VIGS rate (Hamza, 2017).

An alternative approach to analyze the role of proteins is the expression of degradation stable (gain-of-function) protein variants by means of reverse genetics. For example, the functions of different *Aux/IAA* genes from *A. thaliana* (*AtIAA8*, *AtIAA18*, *AtIAA28*) (Rogg *et al.*, 2001; Ploense *et al.*, 2009; Wang *et al.*, 2013), but also from *O. sativa* (*OsIAA1*, *OsIAA11*) were investigated by gain-of-function mutants using a transgenic approach (Nakamura *et al.*, 2006; Song *et al.*, 2009). Since generation of transgenic sugar beet plants is extremely time-consuming because it is a biennial plant, virus mediated expression was chosen to analyse the role on root development of BvIAA2, BvIAA6 and BvIAA28 *in planta*. The wt genes were expressed in *B. vulgaris* and *N. benthamiana* using the viral vector TRV. TRV is a viral vector with a wide host range and a simple ssRNA genome, consisting of two viral RNAs. Moreover, the virus can spread systemically in most plant species and causes only mild viral symptoms, making TRV very useful to study various developmental processes, for example, by VIGs or via heterologous gene expression (Tian *et al.*, 2014; reviewed in Shi *et al.*, 2021). In addition to the wt genes of *BvIAA2*, *BvIAA6* and *BvIAA28*, gain-of-function mutant were chosen to be heterologously expressed. For this purpose, mutations were introduced into domain II of the *Aux/IAA* genes to prevent protein degradation. Unfortunately, expression of *BvIAA2*, *BvIAA6* and *BvIAA28* in *B. vulgaris* could not be performed because of the missing systemic movement of the TRV vector into sugar beet roots. A control with a dsRed labelled full-length infectious TRV clone showed, that most plants did not display fluorescence or only showed systemic infection with RNA1. Therefore, heterologous expression of the Aux/IAA proteins was performed in *N. benthamiana* plants to characterize their effect on root

development. Indeed, expression of either *BvIAA2*, *BvIAA6* or *BvIAA28* in *N. benthamiana* affected root development. Infected plants were characterized by dramatic root mass reduction, as well as an overall root shortening. This shows that the auxin-mediated regulatory pathways are highly conserved across different plant species (*B. vulgaris* versus *N. benthamiana*). Furthermore, the results confirmed that BvIAA2, BvIAA6 and BvIAA28 can alter root development in *N. benthamiana*. Additional phenotypes associated with *BvIAA2*, *BvIAA6* or *BvIAA28* expression included stunting and dwarfing as well as a significant reduction in the number of flowers. The effects on plant development and growth were further enhanced when a gain-of-function mutant of degradation resistant *BvIAA6* was expressed. Unfortunately, the gain-of-function mutants of *BvIAA2* and *BvIAA6* were lethal to the plants. Interestingly, expression of gain-of-function orthologs of these *Aux/IAA* genes from *A. thaliana i.e*. *AtIAA14/SLR*, *AtIAA16*, *AtIAA18*, *AtIAA19* and *AtIAA28* also affected root development, accompanied by reduction in the number of LRs (Fukaki *et al.*, 2002; Uehara *et al.*, 2008; Notaguchi *et al.*, 2012; Rinaldi *et al.*, 2012). Furthermore, expression of degradation stable AtIAA18 even caused a shortening of the internodes, an ortholog from BvIAA2 (Fukaki *et al.*, 2002), the phenotype that was also observed in this study, when *BvIAA2*, *BvIAA6* or *BvIAA28* were expressed in *N. benthamiana*.

Although direct evidence in sugar beet is lacking so far, it can be concluded from the results that BvIAA2, BvIAA6, and BvIAA28 are involved in LR formation, at least in *N. benthamiana*. This is in agreement with previous studies in *A. thaliana* showing that several orthologs of these Aux/IAA proteins are involved in controlling root development and LR formation (Fukaki *et al.*, 2002; Knox *et al.*, 2003; reviewed in Lavenus *et al.*, 2013). Thus, it can be said that p25 interacts with Aux/IAA proteins, which are shown to be involved in root formation and development.

4.5 Pathogenicity of the BNYVV P-type in sugar beet

Another major focus of this thesis was the development of an infectious cDNA clone from the BNYVV P-type. Previous studies with this pathotype were made by using reassortants of BNYVV A-type with RNA3 and 5 from P-type (Liebe *et al.*, 2020), and natural infection using infested field soil (Pferdmenges *et al.*, 2008) or other *Polymyxa*-mediated inoculation systems

(Tamada *et al.*, 2020). Therefore, reassortant effects or secondary infections from non-sterile field soil cannot be excluded. Such risks can only be minimized with a reverse genetic system, which has not been available for the P-type so far. An infectious cDNA clone with all RNA components of the P-type is crucial to make reliable statements about symptom severity and resistance-breaking properties of this pathotype. An interesting point in this regard that was studied in more detail in the past is the impact of the additional RNA5 as a symptom enhancer and causal agent for resistance-breaking properties (Tamada *et al.*, 1989; Liebe *et al.*, 2020). The impact of RNA5 in natural populations on sugar yield loss and resistance-breaking was studied extensively by Tamada in 2020. The experiments showed that in the presence of the J-type RNA5, viral RNA3 accumulation levels increased, which also leads to an enhancement of resistance-breaking properties in *Rz1* resistant sugar beet plants. Most important, however, is the massive yield loss in sugar beets infected with BNYVV isolates carrying RNA5 compared to isolates without RNA5 (Tamada *et al.*, 2020). In these experiments, however, it must be mentioned that the Asian J-type RNA5 variant was used, which is different from the P-type RNA5 variant as already mentioned (Koenig *et al.*, 1997; Miyanishi *et al.*, 1999). In addition, natural populations and a *Polymyxa*-mediated inoculation system were used, which cannot exclude vector effects, secondary infections with other pathogens and population-specific symptoms. Although this represents the natural symptoms that can also be observed in the field, no reliable statements can be made about the symptoms triggered by BNYVV itself. However, since such comprehensive experiments have not yet been carried out with the P-type, it is only possible so far to make assumptions about disease severity and resistance-breaking properties from field observations. A reverse genetic system of BNYVV P-type with an artificial inoculation method that does not rely on natural infection with field soil is therefore very useful to study these aspects in detail.

After the infectivity of the cDNA clone has been successfully proven in three hosts (*N. benthamiana*, *B. macrocarpa* and *B. vulgaris*), foliar symptoms of these hosts caused by the P-type were assessed. Indeed, the foliar symptoms are enhanced in *N. benthamiana* and *B. macrocarpa*. The leaves of the P-type infested *N. benthamiana* plants had much stronger yellowing symptoms compared to the plants infested with the A-type. In the case of *B. macrocarpa*, the entire habitus of the plants was more stunted compared to A-type infested

plants and the leaves displayed reduced size. Most interestingly, however, was the phenotype of infested *B. vulgaris* plants. Compared to the A-type, the P-type also caused more severe foliar symptoms. Although both variants induced the typical yellowing and necrosis along the leaf veins, the P-type does not only cause yellowing along the main veins but yellowing of veins of the entire leaf. Furthermore, a characteristic symptom caused by the P-type were crinkly, deformed leaves. This clearly distinguishes this pathotype from the A-type. Such a foliar symptom enhancement has been described in previous studies (Chiba *et al.*, 2011; Galein *et al.*, 2018; Tamada *et al.*, 2020), but this was the first time that such an intense leaf deformation was observed. This might be due to the fact, that all symptoms have been described from naturally infected plants under field conditions, not from plants, infected with the P-type cDNA clone.

For example, it is known that an inoculum dose as well as environmental conditions optimal for virus replication and systemic movement can increase the aggressiveness of the virus and thus enhancing symptom severity (Tamada *et al.*, 2020; reviewed in Biancardi & Tamada, 2016). Both conditions are fulfilled with the artificial method as well as with the incubation of the inoculated plants in the greenhouse. Foliar symptoms, such as vein-yellowing or necrosis can barely be observed under natural field conditions. Natural transmission using *P. betae* would be a better and more accurate method to simulate field conditions but loading of the vector is very difficult and time consuming, as a virus-free isolate would have to be available first. The used method is much faster and simpler, moreover, the purpose of this study was not to simulate field conditions, but to investigate biological differences between the A- and P-type. It can be summarized that for the first time an infectious cDNA clone of the P-type has been generated. This tool can be used to study the biological properties of this pathotype as well as individual genetic components by means of reverse genetics.

Initially, this system was used to investigate the impact of RNA5 on symptom expression, not only in the leaves but also in the roots. A recent study demonstrated that the J-type RNA5 enhances symptom severity of the virus in natural soil, probably by increasing accumulation of viral RNA3 suggesting synergistic effects of p25 and p26 (Tamada *et al.*, 2020). However, our studies with the P-type lacking RNA5 show that even without RNA5, root and leaf symptoms are induced. Regarding the root symptoms, both, root mass and viral load within the LRs did not differ between infected plants with the P-type with or without RNA5. This

indicates, that RNA5 is not the only causal agent for the characteristic massive LR proliferation which is in accordance with the study performed by Tamada and coworkers in 2020 using natural soil. Another symptom they encountered with BNYVV infection with J-type RNA5 was scab-like symptoms on sugar beet roots. Such an observation was not made in the case of infection with BYNVV P-type, which is due to the fact that the experiments were performed with sterile soil without the natural population of soil-borne phytophatogenic fungi and bacteria. Therefore, it can be assumed that these symptoms, as already speculated by them, are probably caused by secondary infections and are not induced by BNYVV RNA5. Furthermore, no differences in the root phenotype were observed between the P- und the A-type. Both the viral load as well as the other root parameters did not differ from those caused by the A-type, even in the reassortants in which RNA5 of the P-type was added. In summary, although the P-type induces strong root symptoms under greenhouse conditions, no differences were detected compared to the A-type. In addition, RNA5 cannot be confimed as causal agent for the enhanced root symptoms because this RNA can be omitted during infection and BNYVV still causes equally severe root symptoms.

In contrast to the root symptoms, clear differences in foliar symptoms were observed. As mentioned above, the P-type induces foliar symptoms even without RNA5, but the symptom severity is much weaker compared to the variant with RNA5. Furthermore, reassortment trials revealed that this feature cannot be transferred to the A-type. The symptom severity remains unchanged when the A-type is co-inoculated with P-type RNA5 even though it is systemically detectable in the plant. This is consistent with the results of previous studies, where also no differences in viral load or symptom severity were detected by the presence of RNA5 (Liebe *et al.*, 2020; Tamada *et al.*, 2020). On the one hand this could be due to experimental differences as all experiments before were conducted using reassortants or natural infection using infected pre-hosts. On the other hand, it could be explained by the biology of the P-type. For the first time, an infectious P-type cDNA clone was used, amplified from a natural soil population. This allowed for the first time the application of a reverse genetic system with all P-type components. Using this system and a newly developed inoculation method that does not rely on natural transmission by *P. betae*, it was possible to make reliable statements about the biological impacts of the P-type under controlled greenhouse conditions. Even though it is not to be expected that the observed symptomatology also occurs under field conditions,

these results gave new insights into the biological impact of P-type on the plant and possible causes for the enhanced symptom severity.

4.6 The BNYVV P-type overcomes *Rz1* but not *Rz1* + *Rz2* resistance

To ensure economically profitable sugar beet cultivation, control of rhizomania is essential. Since BNYVV can only be controlled by resistant sugar beet varieties so far, constant monitoring of possible resistance braking isolates is of great importance. It is important not only to identify resistance-breaking isolates in field but also to identify molecular causes responsible for resistance breaking. The molecular background of the resistance, *e.g.* the interaction of Avr determinants with resistance genes, offers the possibility to rapidly check BNYVV populations for resistance-breaking as well as to identify potential targets for resistance breeding of *B. vulgaris*. Reverse genetics can be used to specifically mutate single amino acids in the cDNA clone to test their effect on symptom expression or resistance-breaking properties in the viral background. For example, mutation of the tetrad of p25 from ALHG into VLHG in the cDNA clone from the A-type using PCR mutagenesis is leading to *Rz1* resistance-breaking (Liebe *et al.*, 2020). The P-type has been shown to overcome *Rz1*, but not *Rz2* (Pferdmenges *et al.*, 2008; Bornemann *et al.*, 2015; Galein *et al.*, 2018; Tamada *et al.*, 2020). The P-type cDNA clone provides a tool to investigate these resistance-breaking properties in more detail and, for example, to analyse the role of RNA5 in this regard. First, it can be confirmed that the P-type is able to overcome *Rz1* resistance, even though with a significantly lower replication level. This has already been shown in experiments with natural virus populations and vector transmission by *P. betae* (Heijbroek *et al.*, 1999; Pferdmenges *et al.*, 2008; Tamada *et al.*, 2020) or using reassortant experiments (Liebe *et al.*, 2020). Until now, the resistance-breaking properties of the P-type have been linked to RNA5 independently of the p25 tetrad motif (Chiba *et al.*, 2011; Liebe *et al.*, 2020). Next to the reduced viral load in *Rz1* resistant sugar beets without RNA5, the infection rate is also significantly reduced. From these results it can be concluded that p26 might not be the causal agent for the resistance-breaking properties of the P-type in *Rz1* resistant plants. It indicates, that p26 is a pathogenicity factor enhancing the resistance-breaking properties of this pathotype as supposed before (Chiba *et al.*, 2011; Galein *et al.*, 2018). These properties might be mediated by increasing the accumulation of RNA3 (Tamada *et al.*, 2020).

Since the exact mechanism how the P-type can overcome *Rz1* resistance cannot be addressed with the results, only speculations can be made. It could be speculated, that p26 is able suppress the immune reaction of the plant by interacting with the DNA as transcription factor (Link *et al.*, 2005) or by interaction with *Rz1* directly. For the A-type the resistance-breaking properties relies on amino-acid variation within the tetrad as previously described (Acosta-Leal & Rush, 2007; Acosta-Leal *et al.*, 2008; Pferdmenges *et al.*, 2008; Acosta-Leal *et al.*, 2010). However, so far only one variant of the P-type tetrad of RNA3 has been found, suggesting an alternative pathogenicity mechanism. In general, the resistance of the plant as well as resistance-breaking properties of plant viruses is based on the success or failure of the recognition of the Avr determinant and resistance protein (reviewed in Luderer & Joosten, 2001). Perhaps p26 interrupts or interferes with this recognition in case of *Rz1*. Thus, assuming that p25 is the Avr determinant of *Rz1* (Schirmer *et al.*, 2005; Pferdmenges *et al.*, 2008; Liebe *et al.*, 2020), p26 could interact with p25 to interrupt the recognition of p25 by *Rz1*. Due to strong sequence similarities of both pathogenicity factors (e-value: 4 × 10−10, 22% sequence identity, and a 43% positive match in a 217 AA region), it has been hypothesized that both RNAs are the result of a gene duplication event (Simon-Loriere & Holmes, 2013). Together with the fact that self-interaction of P-type p25 (tetrad SYHG) as well as other p25 variants has already been demonstrated (Klein *et al.*, 2007), it could be concluded that interaction of p25 with p26 might be possible as well. Such an interaction could protect the recognition site of p25 thus interrupt the induction of the resistance response. Another way how p26 could mediate *Rz1* resistance-breaking might be the autoactivating capabilities of this protein. The first 17 amino acids of this protein were shown to autoactivate gene transcription (Covelli *et al.*, 2009). It is known, that p26 is localized to the nucleus of infected cells, so an interaction with DNA motifs such as promoters is likely to occur (Link *et al.*, 2005). Consequently, it is possible, that p26 activates or represses gene-expression, which results in promoting *Rz1* resistance-breaking.

In summary, was shown that the P-type has *Rz1* resistance-breaking properties even without RNA5, but these properties are significantly enhanced by RNA5. It remains unclear how the resistance-breaking properties are mediated, although based on these results and other recent studies, synergistic effects of both RNAs can be assumed, leading to efficient resistance-breaking of *Rz1*.

4.7 BNYVV P-type is closely related to the A-type

It is suspected that BNYVV was persistent in native hosts such as other species of the *Amaranthaceae* in East Asia long before sugar beet cultivation began (Chiba *et al.*, 2011). Some herbaceous plants from this plant family, which can be infected with BNYVV transmitted by *P. betae* are *e.g. Atriplex patula*, *Blitum bonus-henricus*, *Chenopodium hybridum* and *Chenopodium polyspermum* (Hugo *et al.*, 1996). But also plant species from other families can serve as alternative hosts such as *Calystegia sepium*, *Capsella bursa-pastoris*, *Centaurea cyanus*, *Convolvulus arvensis*, *Galinsorga parviflora*, *Matricaria inodora* or *Stellana media* (Mouhanna *et al.*, 2008). The native host plant of BNYVV remains unidentified, but the first transmission event probably occurred in China, as this is where the highest diversity of BYNVV exists (Chiba *et al.*, 2011). Presumably, however, the diversity of BNYVV types has already formed in the natural hosts. Evolutionary, the genetic composition as well as sequence similarities yielded the theory that the today known pathotypes arose from one ancestor population carrying five RNAs (Section on Genome organization and BNYVV pathotypes under 1.1.2 and 1.1.3).

The results of the reassortment experiment with A- and P-type highlight the close evolutionary relationship between these pathotypes and confirm the phylogenetic theory of BNYVV (Chiba *et al.*, 2011). For the first time, the ability of the A- and P-type to form viable reassortments in sugar beet were investigated using the cDNA clones of both pathotypes. It has been shown that all reassortants of RNA1-3 were infective and allowed virus replication without affecting the ability of the virus to accumulate in lateral roots and to move systemically. Such reassortants are also possible with the more distantly related BSBMV (Ratti *et al.*, 2009; Laufer *et al.*, 2018a). This demonstrates that the functions of the proteins encoded on RNA1-3 are highly conserved within sugar beet infecting benyviruses. However, the ELISA values are significantly reduced for RNA3 reassortments in contrast to RNA1 and RNA2 reassortments. This indicates that the function of wt RNA3 cannot be fully restored by a substitution of RNA3 from another type. Whether RNA3 reassortments shows an equally strong expression level as the wt remains unclear and should be tested via northern blot. Nevertheless, this difference could be explained by the low sequence identity of RNA3. The ORFs located on RNA1 and RNA2 are more conserved between the different virus types compared to the ORF located on RNA3. RNA1 and RNA2 encode viral house-keeping genes, essential for virus replication,

assembly, cell-to-cell movement and suppression of post transcriptional gene silencing (reviewed in Richards & Tamada, 1992). The introduction of BNYVV resistant varieties has created a selection pressure suggesting an adaption of the virus, especially in the Pithiviers region, an area which has been used intensively as a sugar beet breeding area (Galein *et al.*, 2018; reviewed in Biancardi *et al.*, 2002). The replacement of the highly adapted RNA3 probably caused fitness penalties, explaining the low virus levels of the reassortants. Summing up, the results confirmed that reassortants between the A- and the P-type are possible. Interestingly, both virus types can occur in mixed infections (Yüksel Özmen *et al.*, 2020) but to our knowledge, no natural reassortants of the A- and the P-type have been identified to date. This is probably highly unlikely, as co-infection experiments of the A-type clone indicate that superinfection is minimized when such closely related virus types infect the same plant (co-infection exclusion) (Laufer *et al.*, 2018a). The same observation was made with other populations of identical, but differently labelled potyviruses (*e.g.* PPV or potato virus X) (Dietrich & Maiss, 2003). The exact mechanism behind co- and super-infection exclusion is still unknown, but RNA silencing (Ratcliff *et al.*, 1997) and special viral proteins mediating exclusion (Folimonova, 2012; Bergua *et al.*, 2014) had been proposed as possible mechanisms.

The reason why and how the P-type then spread in a region already infected with BNYVV could also be attributed to the Pithiviers region. As previously mentioned, it is speculated that a *P. betae* strain carrying the P-type was introduced as a J-type from Asia by infested soil (Meulemans *et al.*, 2003). As mentioned above, there is an extremely strong selection pressure due to intense resistance testing of sugar beet. This probably led to an adaptation process of the virus, the evolution of the P-type. As evidenced, the P-type is able to infect *Rz1* resistant sugar beet varieties more effectively and more efficiently with RNA5. This indicates a clear fitness advantage in the Pithiviers region, which enables the P-type to spread. Furthermore, such an adaptation process would explain the sequence differences to J-type RNA5 (Koenig *et al.*, 1997; Miyanishi *et al.*, 1999). In addition to the extensive use of this region for breeding, the spatial separation of the sites where the J-type RNA5 (East Asia) (Tamada *et al.*, 1989; Kiguchi *et al.*, 1996) and the P-type occurs (France, Kazakhstan, the UK and Iran) (Koenig *et al.*, 1997; Koenig & Lennefors, 2000; Harju *et al.*, 2002; Ward *et al.*, 2007; Mehrvar *et al.*, 2009) also leads to changed environmental conditions. These are both factors that enhance the selection of beneficial mutations mediating and at least adapted viruses. Despite

this clear fitness advantage in *Rz1* resistant varieties that are grown in all European sugar beet growing areas, the P-type seems to display only a minor distribution. However, recent studies of BNYVV populations from Japan how that of indicate that the incidence of East Asian BYNVV isolates possessing J-type RNA5 increased in the past decades (Nakagami *et al.*, 2021). Again, this can be explained by the introduction of resistant sugar beet varieties in these areas, as J-type RNA5 has shown similar resistance-breaking properties like P-type RNA5 (Tamada *et al.*, 2020). Such extensive population studies are missing in Europe, but the described results strengthen their importance.

In summary, it can be said that the assumptions of previous phylogenetic studies can be confirmed. It is very likely that an early separation of the B-type occurred during the development of the currently known BNYVV pathotypes. The P-type probably originated from an A-type possessing J-type RNA5, which adapted to the conditions in the Pithiviers region.

5. Future perspectives

Although the interaction of BNYVV with the auxin signaling pathway has been confirmed and two additional Aux/IAA proteins have been identified that interact with p25, BvIAA2 and BvIAA6, the exact mechanism of interaction still remains unclear. To uncover this mechanism, further experiments need to be performed, *e.g.* to exclude possible re-localization of Aux/IAA proteins by p25. To investigate the subcellular localization more precisely, the rapid degradation of the Aux/IAA proteins would have to be inhibited. Either by gain-of-function mutants that were also used for co-IP are used, or the degradation mechanism is inhibited, for example by proteasome inhibitors (MG132) infiltrated directly into the leaf. A possible stabilization of the Aux/IAA proteins by p25 could be tested using a quantitative western blot in combination with an exogenous auxin treatment. If the Aux/IAA proteins are stabilized, their concentration should be higher in a quantitative western blot compared to a variant without p25 after auxin treatment. It might be also possible, that p25 interferes with additional proteins such as ARFs or the SCF^{TIR1} complex or even interacts directly with DNA. To examine these hypotheses, a yeast three-hybrid assay or a chromatin immunoprecipitation (ChIP) assay can be used. The direct effect on root development of BvIAA2, BvIAA6, and BvIAA28 was shown in *N. benthamiana* but not in *B. vulgaris*. Knock-out mutants of the corresponding genes would of course be very helpful, but this is an extremely time-consuming approach in the case of sugar beet since it is a biennial plant. For this reason, the development of an efficient VIGS system might also be very helpful to have a fast and more efficient tool to investigate a multitude of different proteins. It was also found that the interaction is very specific and even single mutations in the proteins lead to a loss of interaction. In the case of p25, changes in the NLS/NES signal do not appear to affect BNYVV infectivity, but the effects on the phenotype remain unclear. It would be very interesting to check what influence such mutations have on the phenotype and whether they cause a fitness penalty in a wt population.

In the second part of this study, an infectious cDNA clone of the P-type was successfully developed for the first time. Using a new vector-free inoculation system, first results have already been obtained regarding symptom expression and resistance-breaking properties of the P-type with the generated clone. In the future, this system will offer the possibility to introduce mutations from wt populations rapidly and test them for biological properties specifically. For example, it has been shown that the additional RNA5 is an *Rz1*

resistance-breaking enhancer, acting synergistically with p25. An interesting point would be, whether the resistance-breaking properties of the P-type RNA5 can be transferred to the A-type or whether the P-type specific p25 tetrad (SYHG) is responsible for the synergism. The exact mode of *Rz1* resistance-breaking could also be further investigated at molecular level. Y2H and ChIP assays could answer the question if p26 interacts with other proteins such as p25 or if p26 directly regulates gene transcription as a transcription factor to inhibit the plant immune response. Luckily, no resistance-breaking of *Rz2* was detected, but the cDNA clone is a very useful tool to investigate different mutations found in wt populations for *Rz2* resistance-breaking monitoring. Using sequence comparisons and reassortant experiments with the A-type, the theory about the phylogenetic relationship of the BNYVV pathotypes could be further confirmed. The results show that the A- and P-type are closely related and form viable RNA1-3 reassortments in sugar beet without significant fitness penalties. Due to the sequence difference between the B- and the P-type, a different reaction would be expected, that reassortants are possible but with stronger fitness penalties. A reassortment of the J-type RNA5 with the P-type RNA5 would be interesting to examine whether both RNAs have the same functions regarding symptom enhancement and resistance-breaking. These are questions which should be addressed in future to understand rhizomania pathogenesis and to control this disease in future.

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8. Curriculum Vitae

Maximilian Martin Müllender

Microbiology, M. Sc.

Personal Details

Gender: Male
Date of Birth: 06 September 1994
Place of Birth: Krefeld, Germany
Nationality: German

Professional Experiences

Since 03/2019	**Graduate Researcher** *Institute of Sugar Beet Research (IfZ), Göttingen, Germany*
08/2017 – 03/2018	**Student Apprentice** *Global Development Fungicides at BASF SE Agricultural Center, Limburgerhof, Germany*

Education

Since 03/2019	**Ph.D.** *Georg-August-University Göttingen/ Institute of Sugar Beet Research (IfZ)* • Title: 'Molecular causes for symptom expression of beet necrotic yellow vein virus in *Beta vulgaris*'
10/2016 – 02/2019	**M. Sc. Microbiology** *Rhenish Friedrich-Wilhelm University, Bonn, Germany*
10/2013 – 09/2016	**B. Sc. Biology** *Rhenish Friedrich-Wilhelm University, Bonn, Germany*

Memberships

Since 2017	Member of the *Deutsche Phytomedizinische Gesellschaft e.V.* (DPG) – *German Society for Plant Protection and Plant Health R. S. (DPG)*

Publications

Muellender, M. M., Mahlein, A.-K., Stammler, G., Varrelmann, M. (2021). Evidence for the association of target-site resistance in *cyp51* with reduced DMI sensitivity in European *Cercospora beticola* field isolates. In *Pest Management Science*, 77(4):1765-1774. doi: 10.1002/ps.6197. https://pubmed.ncbi.nlm.nih.gov/33236506/

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Presentations und Posters

- **Müllender, M.**, Stammler, G., Mahlein, A.-K., Varrelmann, M. (2019, March). Causes and mechanisms for alterations in the sensitivity of *Cercospora beticola* towards DMI fungicides. Jahrestreffen der Arbeitskreise „Mykologie“ und „Wirt-Parasit-Beziehungen“, Kaiserslautern.
- **Müllender, M.** (2019, June). Interaction of Aux/IAA proteins in sugar beet with the viral pathogenicity factor p25 of BNYVV. PSSC - Plant science student conference, Halle.
- **Müllender, M.**, Liebe, S., Varrelmann, M. (2019, October). Interaction of Aux/IAA proteins in sugar beet with the viral pathogenicity factor p25 of BNYVV. International Advances in Plant Virology, poster, Rome, Italy.
- **Müllender, M.**, Varrelmann, M., Stammler, G. (2020, February). Possible causes and mechanisms for alterations in the sensitivity of *Cercospora beticola* towards DMI fungicides. 77. IIRB Congress, poster, Brussels, Belgium.
- **Müllender, M.**, Mahlein, A.-K., Stammler, G., Varrelmann, M. (2020, September). First evidence for target-site resistance in *cyp51* associated with reduced DMI sensitivity in European *Cercospora beticola* field isolates. IIRB Pests & Diseases study group meeting, online.
- **Müllender, M.**, Liebe, S., Varrelmann, M. (2021, April) Interaction of Aux/IAA proteins in sugar beet with the viral pathogenicity factor p25 of BNYVV. AAB Conference: International Advances in Plant Virology, poster, online.
- **Müllender, M.**, Mahlein, A.-K., Stammler, G., Varrelmann, M. (2021, March). First evidence for target-site resistance in *cyp51* associated with reduced DMI sensitivity in European *Cercospora beticola* field isolates. American Society of Sugar Beet Technologists virtual meeting (ASSBT), online.
- **Müllender, M.**, Mahlein, A.-K., Stammler, G., Varrelmann, M. (2021, September). Erster Nachweis für eine Target-Site Resistenz von *Cercospora beticola* gegenüber Azolen – Mögliche Anwendung im Monitoring. 15. Göttinger Zuckerrübentagung, online.
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- **Müllender, M.**, Mahlein, A.-K., Stammler, G., Varrelmann, M. (2021, September). Beweise für die Assoziation von *cyp51* Target-Site-Resistenzen mit reduzierter DMI-Empfindlichkeit in europäischen *Cercospora beticola* Feldisolaten. 62. Deutsche Pflanzenschutztagung, online.

9. Eidesstattliche Erklärung

Hiermit erkläre ich, dass diese Arbeit weder in gleicher noch in ähnlicher Form bereits anderen Prüfungsbehörden vorgelegen hat.

Weiter erkläre ich, dass ich mich an keiner anderen Hochschule um einen Doktorgrad beworben habe.

Köln, den 28.03.2022

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2. Hiermit erkläre ich eidesstattlich, dass diese Dissertation selbständig und ohne unerlaubte Hilfe angefertigt wurde.

Köln, den 28.03.2022

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